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13. ABSTRACT (Maximum 200 Words)

During the last three years, we have demonstrated frequent activation (39%) of PI3K/AKT1 pathway in human primary ovarian cancer. The activation of this pathway is associated with late stage and high grade tumors, indicating that activation of PI3K/AKT1 plays a important role in ovarian tumor progression rather than initiation. Activation of PI3K/AKT1 pathway induces malignant transformation and chemoresistance. Inhibition of this pathway by PI3K inhibitor or dominant negative AKT1 results in apoptosis and cell growth arrest as well as sensitizes ovarian cancer cells to cisplatin-induced cell death. We have also demonstrated that AKT targets ASK1/JNK/p38 pathway to promote cell survival. Further, we documented that farnesyltransferase inhibitor (FTI) and geranylgeranyltransferase I Inhibitors (GGTI) inhibit PI3K/AKT pathway to overcome chemoresistance in human ovarian cancer cells.

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INTRODUCTION:

The purpose of this project is to: 1) Determine the incidence and clinical significance of *PI3K/AKT1* alterations in ovarian cancer; 2) Determine the role of overexpression of active and wild type PI3K and AKT1 in ovarian surface epithelial cell transformation and 3) Determine PI3K and AKT1 as targets for ovarian cancer intervention

BODY:

During the last 3 years, we have accomplished the experiments proposed in grant application. Some of the experiments, such as transgenic mouse, are still under investigation. However, we have extended some of studies to further step based on the scientific nature.

Task 1. Determine the incidence and clinical significance of *PI3K/AKT1* alterations in ovarian cancer.

We have examined 91 ovarian tumor specimens for protein expression and kinase activity of AKT1 and p110, a catalytic subunit of PI3K. Activation of PI3K and AKT1 was observed in 39.3% tumor examined, however, elevated AKT1 protein level was only detected in 8.3% (1, 3; see Appendix). We have also examined the alterations of PTEN, a tumor suppressor gene encoding dual phosphatase that dephosphorylates PI3K products ($PI3,4,5P_3$) and inhibits AKT1 activity (2), in this series of tumors. Three cases showed PTEN down-regulation. These data suggest that PI3K activation is primary reason to result in elevated AKT1 kinase levels in human ovarian cancer. The majority of the tumors with PI3K/AKT1 activation are late stages and high grades indicating that alterations of PI3K/AKT1 are associated with tumor progression rather than initiation.

To further determine the clinical significance of PI3K/AKT1 activation, cisplatin sensitive (A2780-S) and resistance (A2780-CP) ovarian cancer cell lines were stably transfected with constitutively active Akt (Myr-Akt) and DN-Akt, respectively. Cell growth and Tunel assay analyses revealed that Myr-Akt transfected A2780-S became resistant to cisplatin, whereas DN-Akt sensitized A2780-CP cells to cisplatin-inhibited tumor cell growth (3, see Appendix).

Task 2. Determine the role of overexpression of active and wild type PI3K and AKT1 in ovarian surface epithelial cell transformation

It has been demonstrated that wild type AKT1 was unable to transform murine fibroblasts (4). To determine whether activation of AKT1 in human tumors has biological implication, we have introduced HA-tagged constitutively active forms of Akt (Myr-Akt and Akt-E40K), wild type, and myristoylated kinase-inactive mutant (Myr-Akt-K179M) Akt into NIH 3T3 cells individually. After G418 selection, stable clonal cell lines were obtained. Cells transfected with constitutively active forms of Akt, but not myr-Akt-K179M and WT-Akt were morphologically transformed, grew in medium with low serum (0.1%), formed colonies in soft agar suspension, and were highly tumorigenic in nude mice. Tumors were observed 1-3 weeks after the injection of constitutively activated Akt-transfected cells in all mice (1, see appendix). These data suggest that kinase activity of Akt/AKT1 is essential for oncogenic transformation. However, ectopic expression of wild type

and constitutively active p110 and AKT1 did not result in malignant transformation in human ovarian surface epithelial cells (HIOSE 118).

In addition, three founders of ovarian specific promoter (OSP1) driven-myr-AKT1 and –p110 transgenic mice have been established. Two lines from OSP1-myr-AKT1 and OSP1-p110 transgene have been observed for 18 moths. No tumor and abnormal proliferation were observed so far in mouse ovary.

Task 3. Determine PI3K and AKT1 as targets for ovarian cancer intervention

To assess the PI3K/Akt as a potential target for ovarian cancer intervention, several ovarian cancer cell lines with or without PI3K/AKT1 activation were treated with PI 3-kinase inhibitors, wortmannin or LY294002, or vehicle (DMSO) for 12 hours in a medium containing 1% fetal calf serum. Those cell lines exhibiting elevated levels of PI3K/AKT activity underwent apoptosis, whereas no apoptosis was detected in the cell line without PI3K/AKT activation (5, see Appendix). These data indicate that PI3K/AKT1 pathway is an important target for ovarian cancer intervention.

Because activation of PI3K/AKT1 pathway induces ovarian cancer cells resistant to cisplatin and because JNK and p38 MAPKs are required for chemotherapeutic drug- and cellular stress-induced apoptosis (6-9), we have examined whether PI3K/Akt induced chemoresistance is mediated by JNK/p38 pathway in human ovarian cancer cells. Our results showed that cisplatin and TNFα significantly induced kinase activities of JNK and p38 in human A2780-S and A2780-CP ovarian cancer cells (10, 11; see appendix). Expression of constitutively active p100* and Myr-Akt abrogates JNK/p38 activation induced by cisplatin and TNFα, indicating that cisplatin resistance is in some extent mediated by PI3K/Akt inhibition of cisplatin-stimulated JNK/p38 activation. To further define the mechanism of PI3K/Akt inhibition of JNK/p38, we have examined the effect of PI3K/Akt on ASK1 kinase activity, which is a key molecule to activate JNK/p38. We have observed that Akt interacts with and inhibits ASK1 by phosphorylation of Serine-83 of ASK1. Expression of nonphosphoryable ASK1-S83A sensitized A2780 ovarian cancer cells to cisplatin-induced apoptosis and abrogated the inhibitory effects of Akt on JNK/p38 activation, whereas phosphorylation mimic ASK1-S83D rendered cells to resistant to programmed cell death (11, see Appendix). These results indicate that activated PI3K/Akt protection of cells from cisplatin-induced apoptosis is mediated by Akt inhibition of stress kinases and provide the evidence that Akt inhibition of JNK/p38 is via inhibition of ASK1 kinase.

To determine if inhibition of PI3K/Akt pathway sensitizes chemotherapeutic drug-induced programmed cell death in human ovarian cancer cells, cisplatin resistance A2780-CP cells were treated with cisplatin together with or without PI3K inhibitor LY294002, farnesyltransferase inhibitor (FTI) or geranylgeranyltransferase I Inhibitor (GGTI) which have been shown by us to specifically inhibit PI3K/Akt pathway (12, 13). Apoptosis was significantly induced by treating the cells with cisplatin/LY294002, cisplatin/FTI or cisplatin/GGTI as compared to cisplatin alone. We also showed that GGTI or FTI alone was able to induce apoptosis in human ovarian cancer cells with activation of PI3K/Akt (11-13, see Appendix). . These data further indicate that PI3K/Akt pathway is a critical target for ovarian cancer intervention. The reagents targeting PI3K or Akt

could be potential drugs for ovarian cancer treatment, especially in chemoresistant tumors.

In addition, we have developed a new project, i.e., alteration of Aurora-A/BTAK oncogene in human ovarian cancer (14, see Appendix). Total 92 patients with primary ovarian tumors were examined for kinase activity and protein levels of BTAK/Aurora-A. Elevated kinase activity and protein levels of BTAK/Aurora-A were detected in 44 and 52 specimens, respectively.

KEY RESEARCH ACCOMPLISHMENTS:

1. Identification of frequent alterations of PI3K/AKT1 in human primary ovarian cancer which associates with tumor progression rather than initiation.
2. Activation of AKT1 in human ovarian cancer has important biological implication in term of malignant transformation.
3. PI3K/AKT1 pathway is a critical target for anti-tumor drug discovery.
4. Akt inhibition of JNK/p38 by phosphorylation of ASK1 mediates Akt antiapoptotic function
5. Farnesyltransferase and geranylgeranyltransferase I Inhibitors target PI3K/AKT pathway resulting in inhibition of growth and induction of apoptosis in human ovarian cancer.

REPORTABLE OUTCOMES:

Manuscripts:

1. AKT1/PKBα kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am. J. Pathology* 159:431-437, 2001.
2. Phosphatidylinositol-3-OH kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor α (ERα) via interaction between ERα and PI3K. *Cancer Res.*, 61:5985-5991, 2001.
3. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene* 19:2324-2330, 2000.
4. Inhibition of JNK by cellular stress- and tumor necrosis factor α-induced AKT through activation of the NFκB pathway in human epithelial Cells. J Biol Chem. 277(33):29973-2982, 2002.
5. Apoptosis Protein in chemoresistance in Ovarian Cancer: Possible involvement of the PI 3-Kinase/Akt Pathway. *Drug Resist Updat.* 5:131-146, 2002.
6. PI3K/AKT pathway regulates TSC tumor suppressor complex by phosphorylation of tuberin. *J. Biol. Chem.* 277:35364-35370, 2002.
7. AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: Implication of AKT2 in chemoresistance. *J. Biol. Chem.* 278:23432-13440, 2003.

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CONCLUSIONS:

1. Activation of PI3K/AKT1 pathway is a common occurrence in human ovarian cancer.
2. Activation of PI3K/AKT1 results in malignant transformation and contributes to cisplatin resistance in human ovarian cancer.
3. Inhibition PI3K/AKT1 pathway inhibits ovarian cancer cell growth and induces apoptosis.
4. PI3K and Akt are critical targets for ovarian cancer intervention.
5. Akt inhibition of JNK/p38 by phosphorylation of ASK1 mediates Akt-induced chemoresistance.
6. GGTI and FTI could be potential therapeutic reagents to treat human ovarian cancer, especially the tumors resistant to conventional chemotherapeutic drugs.

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1. Sun M, Wang, G, Paciga, JE, Feldman RI, Yuan Z, Ma X, Shelley SA, Jove R, Tsichlis PN, Nicosia SV, Cheng J.Q. AKT1/PKBα kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am. J. Pathology* 159:431-437, 2001.
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Short Communication

AKT1/PKBα Kinase Is Frequently Elevated in Human Cancers and Its Constitutive Activation Is Required for Oncogenic Transformation in NIH3T3 Cells

Mei Sun,* Gen Wang,* June E. Paciga,* Richard I. Feldman,† Zeng-Qiang Yuan,* Xiao-Ling Ma,* Sue A. Shelley,* Richard Jove,* Philip N. Tsichlis,‡ Santo V. Nicosia,* and Jin Q. Cheng*

From the Department of Pathology and Laboratory Medicine and the Molecular Oncology Program, University of South Florida College of Medicine and H. Lee Moffitt Cancer Center, Tampa, Florida; the Cancer Research Department,† Berlex Biosciences, Richmond, California; and the Kimmel Cancer Center,‡ Thomas Jefferson University, Philadelphia, Pennsylvania*

Extensive studies have demonstrated that the Akt/AKT1 pathway is essential for cell survival and inhibition of apoptosis; however, alterations of Akt/AKT1 in human primary tumors have not been well documented. In this report, significantly increased AKT1 kinase activity was detected in primary carcinomas of prostate (16 of 30), breast (19 of 50), and ovary (11 of 28). The results were confirmed by Western blot and immunohistochemical staining analyses with phospho-Ser473 Akt antibody. The majority of AKT1-activated tumors are high grade and stage III/IV (13 of 16 prostate, 15 of 19 breast, and 8 of 11 ovarian carcinomas). Previous studies showed that wild-type AKT1 was unable to transform NIH3T3 cells. To demonstrate the biological significance of AKT1 activation in human cancer, constitutively activated AKT1 (Myr-Akt) was introduced into NIH3T3 cells. Overexpression of Myr-Akt in the stably transfected cells resulted in malignant phenotype, as determined by growth in soft agar and tumor formation in nude mice. These data indicate that AKT1 kinase, which is frequently activated in human cancer, is a determinant in oncogenesis and a potential target for cancer intervention. ***(Am J Pathol 2001, 159:431–437)***

Akt, also known as protein kinase B, represents a subfamily of the serine/threonine protein kinases.[1–5] Akt/AKT1/PKBα signaling has been extensively studied throughout the last 6 years. It has been shown that Akt is activated by a variety of stimuli in a phosphoinositide-3-OH kinase (PI 3-kinase)-dependent manner.[6–9] Activation of Akt by growth factors depends on the integrity of the PH domain, which binds to the PI 3-kinase product, $PI(3,4,5)P_3$, and phosphorylation of Thr-308 and Ser-473 by PDK1 and PDK2/ILK, respectively. In addition, growth factor-induced Akt activation is also mediated by Ras, Src, and Gab1.[10–12] In numerous cell types, it has been shown that Akt induces survival and suppresses apoptosis induced by a variety of stimuli, including growth factor withdrawal and loss of cell adhesion. The mechanisms by which Akt promotes cell survival include phosphorylation of the pro-apoptotic proteins BAD, caspase-9, Forkhead transcription factors, and IκB kinase α, resulting in reduced binding of BAD to Bcl-X_L, inhibition of caspase-9 protease activity, Fas ligand gene transcription, and activation of the nuclear factor-κB cascade.[13–17] Akt has also been shown to inhibit the Raf-MEK-ERK pathway through phosphorylation of Raf-1 in myotubes and overcome constitutively activated MAPK-induced cell-cycle arrest in MCF7 cells.[18,19]

Although Akt/AKT1 is essential for cell survival and anti-apoptosis, alterations of Akt/AKT1 have not been consistently observed in any human malignancy. In fact, amplification of AKT1 has been reported in only a single gastric carcinoma.[20] In this communication, we describe frequent activation of AKT1 in human carcinomas of prostate, breast, and ovary. We also demonstrate the biological significance of AKT1 activation in human cancer by showing that constitutively activated, but not wild-type, Akt is highly oncogenic in NIH3T3 cells.

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M.S. and G.W. contributed equally to this work.

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Materials and Methods

Tumor Specimens, Cell Lines, Transfection, and Transformation Assay

All primary human cancer specimens were obtained from patients who underwent surgery at the H. Lee Moffitt Cancer Center and each sample contained at least 70% tumor cells as was confirmed by histological examination. The tissues were snap-frozen and stored at −70°C. Slides from each case were reviewed for grade and stage following the criteria of the American Joint Committee on Cancer, 1988 edition. NIH3T3 cells were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Transfection was performed with LipofectAMINE PLUS (Life Technologies, Inc., Rockville, MD). Stable clonal cell lines were established after G418 selection. Soft agar suspension and tumorigenesis assays were performed as previously described.[21]

Plasmids

Hemagglutin epitope (HA)-tagged wild-type, constitutively active (Myr-Akt and Akt-E40K), and dominant-negative (kinase-inactive mutant Myr-Akt-K179M) Akt were described previously.[22]

Immunoprecipitation and Western Blotting Analysis

The frozen tissue was lysed by a tissue tearor in a lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mmol/L phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mmol/L benzamidine, 20 mmol/L NaF, 10 mmol/L NaPPi, 1 mmol/L sodium vanadate, and 25 mmol/L β-glycerophosphate. An equal amount of protein was analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4°C for 20 minutes. After removal of the beads by centrifugation, lysates were incubated with the indicated antibody in the presence of protein A-protein G (2:1) agarose beads for 2 hours at 4°C. The beads were washed once with 50 mmol/L Tris-HCl (pH 7.5), 0.5 mol/L LiCl, 0.5% Triton X-10, twice with phosphate-buffered saline (PBS), and once with 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, and 1 mmol/L dithiothreitol, all containing 20 mmol/L β-glycerophosphate and 0.1 mmol/L sodium vanadate. Immunoprecipitates were subjected to *in vitro* kinase assays or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates with phospho-Ser473 Akt (New England Biolabs, Beverly, MA) or anti-AKT1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibody. Detection of antigen-bound antibody was performed with the ECL Western blotting analysis system (Amersham, Arlington Heights, IL).

Immunohistochemistry

Formalin-fixed paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded alcohol to distilled water. The sections were subjected to antigen retrieval by boiling in a microwave for 30 minutes in 0.01 mol/L sodium citrate buffer (pH 6.0) and then exposed to 3% hydrogen peroxide diluted in water for 20 minutes at room temperature to block endogenous peroxidase activity. Sections were incubated in a blocking solution (PBS containing blocking serum) for 20 minutes followed by 20 minutes with an avidin/biotin blocking solution (Vector Laboratories, Burlingame, CA). The primary antibody to phospho-S473 Akt (Upstate Biotechnology, Lake Placid, NY) or PTEN (Upstate Biotechnology) was applied and incubated overnight at 4°C. After incubation, the slides were treated with biotinylated secondary antibody, washed, and treated with streptavidin and biotinylated horseradish peroxidase according to the manufacturer's instruction (Vector Laboratories, Burlingame, CA). After washing, the signal was visualized by diaminobenzidine tetrahydrochloride. A negative control reaction with no primary antibody was always performed alongside the reaction-containing sample.

In Vitro *Protein Kinase Assay*

In vitro Akt kinase assays were performed as previously described.[11] Briefly, the reaction was performed in the presence of 10 μCi of [γ-^{32}P]ATP (New England Nuclear, Boston, MA) and 3 μmol/L of cold ATP in 30 μl of buffer containing 20 mmol/L Hepes (pH 7.4), 10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, and 1 mmol/L dithiothreitol. Histone H_2B was used as the exogenous substrate. After incubation at room temperature for 30 minutes, the reactions were stopped by adding protein-loading buffer, and the products were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

PI 3-Kinase Assay

Anti-p85 (Santa Cruz) antibody was used to immunoprecipitate p110 catalytic subunits of PI 3-kinase from the tumor lysate. The immunoprecipitates were washed once with cold PBS, twice with 0.5 mol/L LiCl, 0.1 mol/L Tris (pH 7.4), and finally with 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid. The presence of PI 3-kinase activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mmol/L HEPES (pH 7.4), 10 mmol/L $MgCl_2$, 50 μmol/L ATP, 20 μCi [γ-^{32}P]ATP, and 10 μg L-α-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P_2; Biomol, Plymouth Meeting, PA) or L-α-phosphatidylinositol-4-phosphate (Sigma Chemical Co., St. Louis, MO) for 20 minutes at 25°C. The reactions were stopped by adding 100 μl of 1 mol/L HCl. Phospholipids were

extracted with 200 μl of $CHCl_3CH_3$/MeOH. Phosphorylated products were separated by thin-layer chromatography as previously described.[23] The conversion of PI-4,5-P_2 to PI-3,4,5-P_3 and PI-4-P_1 to PI-3,4-P_2 was determined by autoradiography and quantitated by using a PhosphorImager.

Results

Frequent AKT1 Activation in Human Cancer

Despite the fact that the PI 3-kinase/Akt/AKT1 pathway is essential for cell survival and anti-apoptosis, consistent alterations of AKT1 in human primary tumors have not been well documented. We have previously examined AKT1 and AKT2 alterations at the DNA and/or mRNA levels in more than 100 cancer cell lines (including NCI 60 cancer cell lines screen) and more than 300 primary tumors from various organs. Amplification and/or overexpression of AKT2 were observed in 15 to 25% of ovarian and pancreatic tumors examined,[2,24,25] whereas no AKT1 alteration at the DNA or mRNA level was detected (J. Q. Cheng and J.R. Testa, unpublished data). The essential role of AKT1 kinase in cell survival prompted us to examine if AKT1 kinase activity is elevated in human cancer. We first examined whether anti-AKT1 antibody (D-17, Santa Cruz) specifically recognizes AKT1. HEK293 cells were transiently transfected with HA-AKT1, HA-AKT2, or HA-AKT3, lysed, and immunoprecipitated with monoclonal anti-HA antibody. The immunoprecipitates were separated and detected with AKT1 antibody. Figure 1A showed that anti-AKT1 antibody only reacted with HA-AKT1. We next immunoprecipitated AKT1 from lysates prepared from frozen tumors of prostate, breast, and ovary with anti-AKT1 antibody. The AKT1 immunoprecipitates were subjected to *in vitro* kinase assays. Significantly increased AKT1 kinase activity was observed in 16 of 30 prostate adenocarcinomas, 19 of 42 ductal breast cancers, and 11 of 23 ovarian serous adenocarcinomas. No elevated AKT1 activity was detected in eight lobular breast carcinomas, three endometrioid, and two borderline ovarian cancers examined, implying that alteration of AKT1 kinase level primarily involves ductal breast and serous ovarian carcinomas. Moreover, we observed that the majority of AKT1 activated-tumors are high grade and stage III/IV (Table 1), suggesting that activation of AKT1 plays an important role in tumor progression rather than initiation.

Table 1. Frequencies of AKT1 Activation and Tumor Stage and Grade

		Kinase activity		
	n	Normal	High	*P* value
Stage				
Prostate cancer				
T1–T2	12	9	3	0.013§
T3–T4	18	5	13	
Breast cancer				
I–II	19	15	4	0.038§
III–IV	31	16	15	
Ovarian cancer				
I–II	9	7	2	0.155
III–IV	19	10	9	
Grade				
Prostate cancer				
<7*	16	10	6	0.055
>7	14	4	10	
Breast cancer				
1–2†	16	14	2	0.009§
3‡	34	17	17	
Ovarian cancer				
1–2†	11	8	3	0.186
3‡	17	9	8	

*Gleason score.
†Well (1) or moderately (2) differentiated.
‡Poorly differentiated.
§$P < 0.05$.

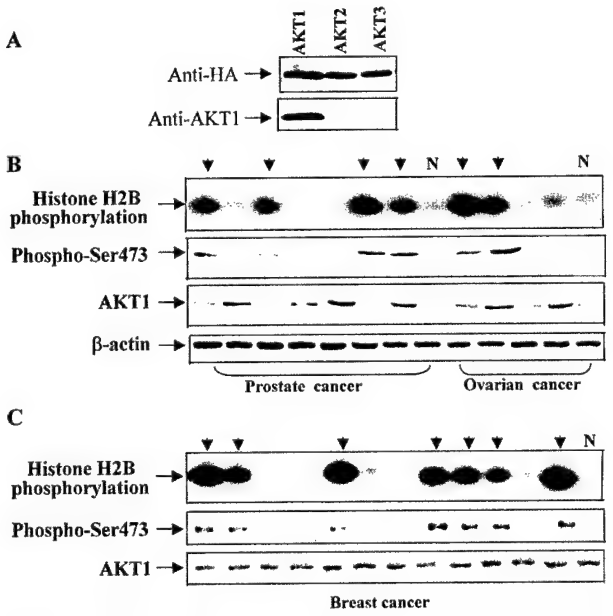

Figure 1. Activation of AKT1 in human cancers. **A:** Western blot. HEK293 cells were transiently transfected with HA-AKT1, HA-AKT2, or HA-AKT3 expression construct, lysed, immunoprecipitated with anti-HA antibody, and detected with anti-HA (**top**) or anti-AKT1 antibody (**bottom**). The top panels of **B** and **C** are *in vitro* kinase assays of AKT1 immunoprecipitates from frozen tumor specimens of prostate and ovary (**B**), and breast (**C**). AKT1 kinase levels were highly elevated in cases indicated by **arrows**. Normal tissue lysates (N) from prostate, ovary, and breast were used as controls. The second panels of **B** and **C** are Western blot analyses of AKT1 immunoprecipitates probed with anti-phospho-Ser473 Akt antibody. The cases exhibiting elevated AKT1 kinase activity displayed phosphorylation bands. The third and fourth panels of **B** and bottom panel of **C** are Western blot analyses with anti-AKT1 and anti-β-actin antibodies.

It has been shown that phosphorylation of threonine-308 and serine-473 is required for activation of AKT1 and that phospho-Ser473 Akt antibody recognizes only the phosphorylated/active form of Akt.[9] To confirm the results obtained from *in vitro* kinase assays, we performed Western blotting analysis with this antibody. To avoid the possibility of cross-reaction of the phospho-Ser473 antibody with other isoforms of Akt/PKB family, the tissue lysates were first immunoprecipitated with the specific anti-AKT1 antibody. The AKT1 immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with anti-phospho-Ser473 antibody. Phosphorylated AKT1 was detected only in the

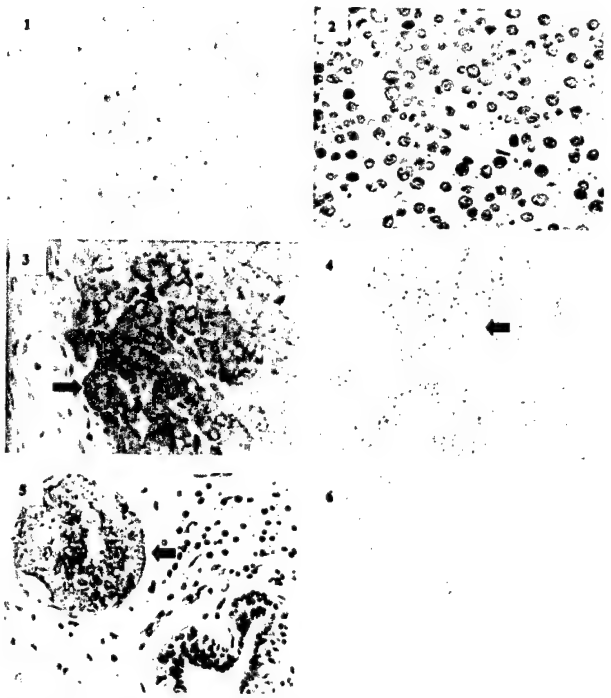

Figure 2. Phospho-AKT1 was detected in tumor cells and located only in cytoplasm. Immunochemical staining of the paraffin sections prepared from serum-starved (**1**), EGF-stimulated MCF7 cells (**2**), and adenocarcinomas of ovary (**3**), prostate (**4**), and breast (**5** and **6**) with phospho-S473 Akt antibody. Strong staining was observed in EGF-stimulated MCF7 cells and ovarian, breast, and prostate tumor cells, indicated by **black arrows**. No immunoreaction was detected in unstimulated MCF7 cells (**1**) normal prostate gland and ductal epithelial cells (**4** and **5**, **white arrow**), and the breast tumor without elevated AKT1 activity (**6**).

tumors with elevated AKT1 kinase activity (second panels of Figure 1, B and C).

To further demonstrate AKT1 activation and determine whether activated AKT1 is derived from tumor cells or stromal tissues in the tumor specimens with elevated AKT1 activity identified by *in vitro* kinase assay, immunohistochemical staining of tumor paraffin sections was performed with phospho-Ser473 Akt antibody. We first demonstrated that the phospho-Ser473 Akt antibody is capable of recognizing phosphorylated AKT1 by immunostaining paraffin sections prepared from serum-starved and serum-starved/EGF-stimulated MCF7 cells (Figure 2, panels 1 and 2). Phosphorylation status of AKT1 in these cells was confirmed by Western blot analysis with phospho-S473 Akt antibody (data not shown). The tumor paraffin sections from the 16 prostate, 19 breast, and 11 ovarian tumor specimens with elevated AKT1 kinase activity strongly immunoreacted with phospho-Ser473 Akt antibody (Figure 2, panels 3 to 5), whereas no immunostaining was observed in normal tissues and the tumor samples without increased AKT1 activity (Figure 2, panel 6). Interestingly, phosphorylated AKT1 was located in the tumor cell membrane and cytoplasm but not the nucleus, which is in conflict with the previously reported observation that activated Akt could translocate to the nucleus in ectopically Akt-overexpressing cells.[26,27] Our data also showed that overexpression of GFP-tagged AKT1 translocates to the nucleus in NIH3T3 cells after IGF-1 stimulation (data not shown),

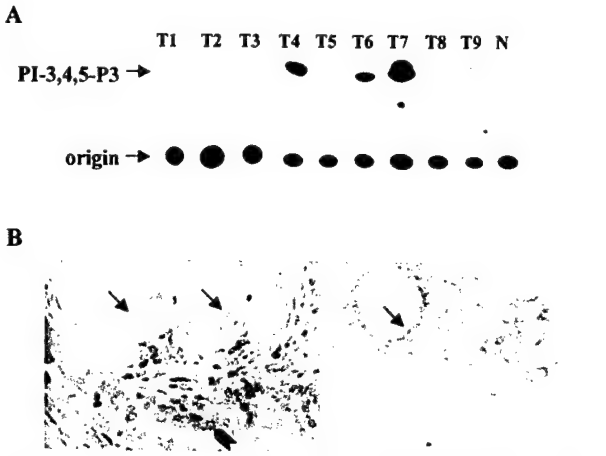

Figure 3. Activation of PI 3-kinase or down-regulation of PTEN in human tumors. **A:** *In vitro* PI 3-kinase assay of the anti-p85 immunoprecipitates from nine tumor specimens that exhibit elevated AKT1 kinase activity. Elevated levels of PI 3-kinase activity were detected in cases 4, 6, and 7. **B:** Immunostaining of paraffin sections of prostate adenocarcinomas with anti-PTEN (**left**) and anti-phospho-Ser473 Akt (**right**) antibodies. PTEN is negative in tumor cells (**black arrows**), but positive in hyperplastic glands (**arrowhead**). However, phosphorylation of Akt was only detected in tumor cells (**black arrow**).

indicating that activated Akt in the primary tumor cells could have a different subcellular localization from the cells overexpressing exogenous Akt.

Multiple Mechanisms Resulted in AKT1 Activation

Because AKT1 kinase activity is regulated positively by PI 3-kinase and negatively by *PTEN/MMAC1*,[28] we examined PI 3-kinase activity and PTEN expression in the tumors exhibiting AKT1 activation (Figure 3). Elevated PI 3-kinase activity was observed in 7 of the 19 breast and 5 of the 11 ovarian carcinomas, but in none of the prostate tumors that exhibit AKT1 activation. Immunohistochemical staining revealed no PTEN expression in 10 of 16 prostate and 2 of 11 ovarian cancer specimens with elevated AKT1 activity, whereas all breast carcinomas that showed AKT1 activation expressed PTEN. Absence of PTEN is well correlated with positive staining of phosphorylated AKT1 on the tumor tissue sections (Figure 3 and data not shown). In addition, we performed single-strand conformational polymorphism/sequencing analyses in AKT1-activated tumor specimens that have neither PI 3-kinase nor PTEN alteration. No AKT1 mutation was detected, implying that there are other mechanisms leading to AKT1 activation in the specimens without alterations of either PI 3-kinase or PTEN, which needs further investigation.

Constitutively Active Forms of Akt/AKT1 Are Highly Oncogenic in NIH3T3 Cells

We and others previously demonstrated that overexpression of wild-type of Akt (WT-Akt) is unable to transform NIH3T3 cells.[21,29] To determine whether activation of AKT1 in human tumors has biological implication, we

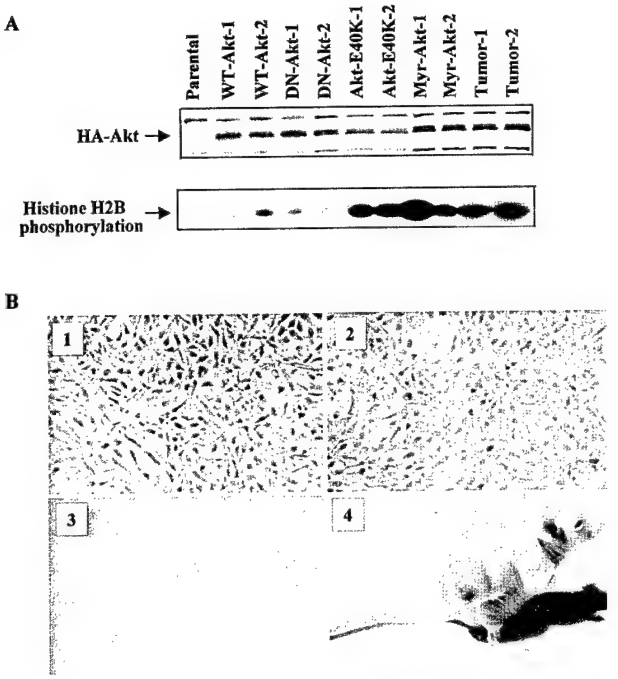

Figure 4. Constitutively activated AKT1 transforms NIH3T3 cells. **A:** Western blot (**top**) and *in vitro* kinase assay (**bottom**) analyses of Akt expression and kinase activity in stably transfected clones and a tumor sample from nude mice. For Western blot analysis, the lysates were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to membrane, and detected with anti-HA antibody. For assay of kinase activity, immunoprecipitation was performed with anti-HA antibody and the HA-Akt immunoprecipitates were subjected to *in vitro* kinase assay using histone H2B as substrate. **B:** The morphology of constitutively active Akt-transfected NIH3T3 cells (**2**) is more rounded and larger than WT-Akt-transfected cells (**1**). Constitutively activated Akt-transfected cells grew on soft agar (**3**) and formed tumors in nude mice (**4**).

have introduced HA-tagged constitutively active forms of Akt (Myr-Akt and Akt-E40K), wild-type, and myristoylated kinase-inactive mutant (Myr-Akt-K179M) Akt into NIH3T3 cells individually. After G418 selection, five stable clonal cell lines from each transfection were obtained. Western blot and *in vitro* kinase analyses revealed that all of the clonal cell lines express Akt protein (Figure 4A). High levels of kinase activity were detected in constitutively active Akt (Myr-Akt and Akt-E40K)-transfected clonal cell lines. Figure 4A shows Akt expression and kinase activity in two clonal cell lines from each transfection. Cells transfected with constitutively active forms of Akt, but not Myr-Akt-K179M and WT-Akt were morphologically transformed, grew in medium with low serum (0.1%), formed colonies in soft agar suspension, and were highly tumorigenic in nude mice (Figure 4B). Tumors were observed 1 to 3 weeks after the injection of constitutively activated Akt-transfected cells in all mice, except clone 2 of Akt-E40K cells (Figure 4 and Table 2). Although vector alone and WT-Akt-transfected NIH3T3 cells developed tumors in 1 of 25 and 2 of 25 mice, respectively, all of these tumors were detected after 38 days (Table 2). In addition, high levels of Myr-AKT1 protein and kinase activity were observed in dissected tumors from Myr-Akt nude mice but not in the tumors from vector or WT-Akt mice (Figure 4A). These data suggest that kinase activity of Akt/AKT1 is essential for oncogenic transformation in NIH3T3 cells.

Table 2. Tumorigenicity of Akt and Control Transfectant NIH3T3 Cell Lines

Clonal cell lines	Soft agar growth*	Tumorigenicity in nude mice†	Latency (days)
Akt-E40K 1	+++	5/5	14–18
Akt-E40K 2	++	4/5	17–22
Akt-E40K 3	+++	5/5	15–21
Akt-E40K 4	+++	5/5	13–18
Akt-E40K 5	+++	5/5	13–18
Myr-Akt 1	+++	5/5	11–14
Myr-Akt 2	+++	5/5	8–10
Myr-Akt 3	+++	5/5	10–11
Myr-Akt 4	+++	5/5	7–10
Myr-Akt 5	+++	5/5	8–11
Myr-Akt-K179M (1–5)	–	0/25	
Vector control (1–5)	–	1/25	40
WT-Akt (1–5)	–	2/25	38–42

*Number of colony/60 mm plate: 1–10, +; 11–30, ++; >30, +++.
†The number of injected cells: 1×10^6/mouse.

Discussion

Activation of oncogenic signaling proteins, such as Stat3, mitogen-activated protein kinase, and p110α, has been demonstrated in a number of different tumors.[30–32] Constitutively activated Stat3 and PI 3-kinase are able to induce malignant transformation.[33,34] Recent studies showed that among the most critical tumor-cell survival pathways are those mediated by the Akt/AKT1 kinase.[6–9] In this report, we demonstrate frequently elevated AKT1 kinase activity and phosphorylation of AKT1 in human carcinomas of breast, prostate, and ovary. Moreover, we have also shown that constitutively activated, but not wild-type, AKT1 is highly tumorigenic in NIH3T3 cells. Furthermore, the majority of AKT1-activated tumors are high grade and stage III/IV. These results indicate that activation of AKT1 is a common occurrence in human cancer, especially in more advanced tumors.

We previously demonstrated that overexpression of wild-type AKT2, but not Akt/AKT1, in NIH3T3 cells resulted in malignant transformation.[21] Ahmed and colleagues[29] also showed that Akt is not tumorigenic when overexpressed in the nontumorigenic rat T-cell lymphoma cell line 5675. In contrast, *v-akt*-expressing 5675 cells were highly tumorigenic. Because v-*akt* arose by an in-frame fusion of the viral *Gag* and *Akt*, the oncogenic difference between v-akt and wild-type Akt/AKT1 may be because of myristoylation of the amino-terminus of v-akt.[29,35] Several lines of evidence show that attachment of a membrane-targeting sequence (myristoylation/palmitoylation) to the amino-terminus of AKT1/Akt is sufficient to induce its maximal activation and that the PH domain of Akt is required for its membrane translocation and activation.[9] We demonstrated, in this study, that overexpression of constitutively active forms of *Akt* (Myr-Akt and Akt-E40K) in NIH3T3 cell leads to oncogenic transformation, which supports the results obtained from chicken embryo fibroblasts and Rat1 cells.[36,37] Taken collectively, these data suggest that the kinase activity of Akt contributes to the control of cell malignant transformation and that elevated AKT1 kinase activity plays an

important role in development and/or progression of a subset of human cancers.

Previous studies have demonstrated that all of the tumor-associated *PTEN* mutants that have been biochemically characterized result in activation of AKT1.[28] Recently, elevated PI 3-kinase activity has been observed in human ovarian cancer.[32,38] As discussed above, Ras, Src, and Gab1 mediate growth factor signals to activate the PI 3-kinase/Akt pathway.[10–12] Therefore, activation of AKT1 in human cancer could result from Ras mutation, overexpression/active mutation of growth factor receptor(s), AKT1 mutation, and Src activation. In the present report, we showed that the majority of cases with AKT1 activation had either PTEN down-regulation or PI 3-kinase activation, dependent on the tumor type. Activation of PI 3-kinase was frequently detected in breast and ovarian, but not prostate, carcinomas, whereas the absence of PTEN protein was observed in some prostate and ovarian carcinomas. However, missense mutations of *PTEN*, although uncommon, cannot be ruled out by immunohistochemistry, because they result in formation of full-length PTEN proteins that may be immunostained by anti-PTEN antibody. Nevertheless, no AKT1 mutation was found in the tumors examined, indicating that elevated AKT1 activity in human cancer results from alterations of upstream regulators of AKT1.

Amplification and/or overexpression of AKT2, but not AKT1, have been detected in a subset of human ovarian, pancreatic, and breast cancers,[2,24,25] suggesting that AKT2 may play a more important role in human malignancy. In the present study, frequent activation of AKT1 kinase in human cancers and malignant transformation resulting from expression of constitutively activated AKT1 provide the first evidence that AKT1 could have a role similar to that of AKT2 in human cancer. Comparison of the AKT and AKT2 protein and/or kinase levels in the same tumor will provide valuable information to better understanding of the importance of AKT1 and AKT2 in human malignancy. Expression of AKT1 and AKT2 protein and alterations of AKT2 at the kinase level in these series of specimens are currently under investigation.

Subcellular localization of activated AKT1 is controversial.[9] Early studies on the subcellular localization of Akt/AKT1 revealed that, whereas c-akt is primarily cytosolic, v-akt is distributed equally between the cell membrane, the cytoplasm, and the nucleus in NIH3T3 cells.[29] Recent studies showed that nuclear translocation of AKT1 and AKT2 in HEK293 and HeLa cells follows in short succession the insulin-induced translocation of AKT1 and AKT2 proteins to the cell membrane.[26,27] However, all of these studies were performed in AKT1- or AKT2-transfected cells. In this report, we demonstrated that activated AKT1 in human primary tumors is distributed only in the plasma membrane and the cytosol, suggesting that activated endogenous AKT1 may not translocate to nucleus. In addition, we noted that immunoreaction to the phospho-Ser-473 antibody in tumor specimens is less strong, which could be because of either weak epitope of single phosphopeptide or epitope masking in paraffin section.

In summary, the data presented in this report showed that AKT1 kinase is frequently activated in human prostate, breast, and ovarian carcinomas. Elevated AKT1 kinase is an essential requirement for its oncogenic activity. These results provide the basis for understanding how the Akt pathway contributes to human oncogenesis. Further studies are required to determine the clinicopathological significance of AKT1 activation and to examine if overexpression of constitutively activated AKT1 develops prostate, breast, and ovarian tumors in transgenic mouse models using tissue-specific promoters.

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Advances in Brief

Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, Activated in Breast Cancer, Regulates and Is Induced by Estrogen Receptor α (ERα) via Interaction between ERα and PI3K[1]

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Abstract

We have shown previously that the AKT2 pathway is essential for cell survival and important in malignant transformation. In this study, we demonstrate elevated kinase levels of AKT2 and phosphatidylinositol-3-OH kinase (PI3K) in 32 of 80 primary breast carcinomas. The majority of the cases with the activation are estrogen receptor α (ERα) positive, which prompted us to examine whether AKT2 regulates ERα activity. We found that constitutively activated AKT2 or AKT2 activated by epidermal growth factor or insulin-like growth factor-1 promotes the transcriptional activity of ERα. This effect occurred in the absence or presence of estrogen. Activated AKT2 phosphorylates ERα *in vitro* and *in vivo*, but it does not phosphorylate a mutant ERα in which ser-167 was replaced by Ala. The PI3K inhibitor, wortmannin, abolishes both the phosphorylation and transcriptional activity of ERα induced by AKT2. However, AKT2-induced ERα activity was not inhibited by tamoxifen but was completely abolished by ICI 164,384, implicating that AKT2-activated ERα contributes to tamoxifen resistance. Moreover, we found that ERα binds to the p85α regulatory subunit of PI3K in the absence or presence of estradiol in epithelial cells and subsequently activates PI3K/AKT2, suggesting ERα regulation of PI3K/AKT2 through a nontranscriptional and ligand-independent mechanism. These data indicate that regulation between the ERα and PI3K/AKT2 pathway (ERα-PI3K/AKT2-ERα) may play an important role in pathogenesis of human breast cancer and could contribute to ligand-independent breast cancer cell growth.

Introduction

Breast cancer development and tumor growth are strongly associated with estrogens. The binding of an estrogen molecule to the ERα[3] induces a cascade of events, including the release of accessory proteins (*e.g.*, the heat-shock proteins), increased nuclear retention, DNA binding, and the transcription of estrogen-responsive genes, such as cyclin D1, c-myc, cathepsin D, and transforming growth factor-α that are known to stimulate mammary cell proliferation (1). ERα is a member of a superfamily of nuclear receptors that act as transcription factors when bound to specific lipophilic hormones. In common with other steroid hormone receptors, the ERα has a NH_2-terminal domain with a hormone-independent transcriptional activation function (AF-1), a central DNA-binding domain, and a COOH-terminal ligand-binding domain with a hormone-dependent transcriptional activation function (AF-2; Refs. 2, 3). In addition to its ligand, estradiol, the ERα is also activated by several nonsteroidal growth factors including EGF and IGF1 through their cell membrane receptors and cytoplasmic signaling pathways such as MAPK signal transduction pathway (3, 4). Because of the role of ERα in promoting the growth and progression of breast cancers, considerable efforts are devoted to development of reagents to functionally inactivate ERα, so as to inhibit ERα-mediated gene expression and cell proliferation. Antiestrogens such as tamoxifen and ICI 164,384 antagonize the effects of estrogens by competing with estrogen for binding to ERα. Tamoxifen or its derivative 4-hydroxytamoxifen inhibits transcriptional activation by AF-2 but not AF-1 (5). ICI 164,384, on other hand, is a complete antagonist that blocks transcriptional activation by both AF-1 and AF-2 (6). However, approximately one-third of ERα-positive breast cancers fail to respond to antiestrogen treatment, which is thought to result from growth factor-induced ERα activity through activation of protein kinases resulting in phosphorylation of ERα (7).

It has been well documented that phosphorylation of ERα is essential for the activation of ERα after stimulation with its ligand and nonsteroidal growth factors (EGF and IGF1). The phosphorylation of ERα is observed at both serine and tyrosine residues. The serine residues are the predominant modified amino acids present in ERα, and four of these (Ser-104, Ser-106, Ser-118, and Ser-167) are clustered in the NH_2 terminus within the AF-1 region. Phosphorylation of ERα at Ser-118 is mediated by the Ras/MAPK pathway; therefore, activation of the MAPK pathway enables ligand-independent transactivation of ERα (4). There is evidence showing that Ser-167 is phosphorylated by several protein kinases, including casein kinase II and $pp90^{rsk1}$, which is important for DNA binding and transcriptional activation (8, 9). Phosphorylation of ERα on tyrosine 537, which is required for ERα dimerization and transactivation, by Src family tyrosine kinases *in vitro* has also been demonstrated. Moreover, protein kinase A has been shown to phosphorylate ERα at Ser-236 and regulate its dimerization (10).

In addition, recent studies (11) demonstrated that plasma membrane ERα plays a crucial role in transducing cellular signals. It has been convincingly shown that ERα activates G-protein-coupled receptor leading to the modulation of downstream pathways that have discrete cellular actions including membrane K^+ and Ca^{2+} channel activation and induction of protein kinase C and protein kinase A kinase activity (11). A recent study (12) demonstrated that estrogen activates p38 MAPK, resulting in the activation MAPK-protein kinase-2 and subsequent phosphorylation of heat shock protein 27. ERα has been also shown to interact with IGF1R and induce IGF1R and extracellular signal-regulated kinase activation (13).

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[3] The abbreviations used are: ERα, estrogen receptor α; PI3K, phosphatidylinositol-3-OH kinase; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; EGF, epidermal growth factor; IGF1, insulin-like growth factor-1; GST, glutathione *S*-transferase; HEK, human embryonic kidney.

Akt, also called protein kinase B, has been identified as a direct target of PI3K (14). All of the three members, Akt/AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ, of this family are activated by growth factors in a PI3K-dependent manner (14–16). Numerous studies (17) showed that the Akt pathway is critical for cell survival by phosphorylation of a number of downstream proteins including BAD, caspase-9, Forkhead transcription factors, IKKα, Raf, and p21-activated protein kinase. Among Akt family members, AKT2 has been shown to be predominantly involved in human malignancies such as ovarian and pancreatic cancers (18–20). In this study, we demonstrate frequent activation of AKT2 and PI3K in human breast cancer. AKT2 phosphorylates ERα at Ser-167 and activates ERα-mediated transcription in a PI3K-dependent manner. ERα binds to the p85α subunit of PI3K in epithelial cells and activates the PI3K/AKT2 pathway in an estrogen-independent manner.

Materials and Methods

Tumor Specimens, Cell Lines, and Transfection. All of the 80 primary human breast cancer specimens were obtained from patients who underwent surgery at H. Lee Moffitt Cancer Center, and each sample contained at least 70% tumor cells, as was confirmed by microscopic examination. The tissues were snap-frozen and stored at −70°C. ERα-negative epithelial HEK293 and COS7 cells and ERα-positive MCF7 and BG-1 cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS. The cells were seeded in 60-mm Petri dishes at a density of 8×10^5 cells/dish. Transfections were performed by calcium phosphate DNA precipitation or Lipofectamine Plus (Life Technologies, Inc.).

Immunoprecipitation and Western Blotting Analysis. The cells and frozen tumor tissues were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (volume for volume) glycerol, 1% NP40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM $NaPP_i$, 1 mM sodium vanadate, and 25 mM β-glycerol phosphate. Lysates were centrifuged at 12,000 × *g* for 15 min at 4°C before immunoprecipitation or Western blotting. The protein concentration in each tissue lysate was measured, and an equal amount of protein was analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4°C for 20 min. After removal of the beads by centrifugation, lysates were incubated with anti-AKT2 (Upstate Biotechnology) antibody in the presence of 30 μl of protein A-protein G (2:1) agarose beads for 2 h at 4°C. The beads were washed once with 50 mM Tris-HCl (pH 7.5)-0.5 M LiCl-0.5% Triton X-10, twice with PBS, and once with 10 mM Tris-HCl (pH 7.5)-10 mM $MgCl_2$-10 mM $MnCl_2$-1 mM DTT, all containing 20 mM β-glycerol phosphate and 0.1 mM sodium vanadate. Immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein phosphorylation and expression were determined by probing Western blots of immunoprecipitates with anti-phospho-Akt-Ser473 (Cell Signaling) or anti-AKT2 antibody. Detection of antigen-bound antibody was carried out with the enhanced chemiluminescence Western Blotting Analysis System (Amersham).

***In Vitro* Protein Kinase Assay.** Akt kinase assay was performed as described previously (15). Briefly, the reaction was carried out in the presence of 10 μCi of [γ-^{32}P]ATP (NEN) and 3 μM cold ATP in 30 μl of buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM DTT using histone H2B as substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding protein-loading buffer, and the mixture was separated in SDS-PAGE gels. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Phosphorimager (Molecular Dynamics).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven after dewaxing and rehydration. The Vectastain ABC Kit for sheep IgG (Vector Laboratories) was used to immunostain the tissue sections with phospho-S473 Akt antibody (catalogue number 06-801-MN; Upstate Biotechnology). Endogenous peroxidase and biotin were blocked, and sections were incubated 1 h at room temperature with a 1:250 dilution of antibody to phospho-S473 Akt. The remainder of the staining procedure was performed according to the manufacturer's instructions using diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin for counterstaining. Primary antibody was replaced with an equal concentration of nonimmune sheep IgG on negative control sections.

PI3K Assay. PI3K was immunoprecipitated from the tumor tissue lysates with pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4), and finally with 10 mM Tris, 100 mM NaCl, 1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP, 20 μCi [γ-^{32}P]ATP, and 10 μg of L-a-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P_2; BIOMOL) for 20 min at 25°C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of $CHCl_3$/methanol. Phosphorylated products were separated by TLC as described previously (21). The conversion of PI-4,5-P_2 to PI-3,4,5-P_3 was determined by autoradiography and quantitated by using a Phosphorimager. Average readings of the kinase activity 3-fold higher than that in normal ovarian tissue was considered as elevated PI3K activity.

Expression Constructs and GST Fusion Protein. HA epitope-tagged constitutively active, wild-type, and dominant-negative AKT2 were prepared as described previously (21). The p110α and p85α of PI3K expression constructs were gifts from Dr. Julian Downward (Imperial Cancer Research Fund, London, United Kingdom). The mammalian expression construct of ERα-S167A was kindly provided by Dr. Benita S. Katzenellenbogen (University of Illinois, Urbana, IL). The GST-ERα and GST-ERα-S167A were created by PCR and inserted into pcDNA3 and pEGX-4T (Pharmacia) vectors, respectively. GST-ERα fusion proteins were purified as described previously (21).

***In Vivo* [^{32}P]P_i Cell Labeling.** Transfected COS7 and nontransfected MCF7 cells were labeled with [^{32}P]P_i (0.5 mCi/ml) in MEM without phosphate, serum, and phenol red for 4 h and lysed. ERα was immunoprecipitated with monoclonal anti-ERα or anti-myc antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated ERα was detected by autoradiography and quantitated by using Molecular Dynamics Phosphorimager with ImageQuant software.

Reporter Assay. HEK293 and MCF7 cells (8×10^5) were seeded in a 60-mm plate. The cells were cotransfected with the luciferase reporter plasmid (2ERE-MpG12), wild-type, constitutively active, or dominant-negative AKT2 and ERα, as well as pCMV-βgal plasmid as an internal control. The amount of DNA in each transfection was kept constant by the addition of empty pcDNA3 vector. Luciferase and β-galactosidase activities were determined 48 h after transfection according to the manufacturer's procedure (Promega). Luciferase activity was corrected for transfection efficiency by using the control β-galactosidase activity. All of the experiments were performed in triplicate from independent cell cultures.

Results and Discussion

Frequent Activation of AKT2 in Breast Carcinoma. We have demonstrated previously (15, 20) that AKT2, like AKT1, is activated by a number of mitogenic growth factors in a PI3K-dependent manner and that AKT2 kinase activity is frequently elevated in human ovarian tumors. To examine whether AKT2 is activated in human primary breast cancer, we performed *in vitro* kinase assays in 80 human breast carcinoma specimens, including 58 ductal infiltrating adenocarcinomas, 16 lobular carcinomas, and six mixed tumors. Lysates from tumor specimens were incubated with anti-AKT2 antibody, which specifically reacts with AKT2 (20). The immunoprecipitates were subjected to *in vitro* kinase assay using histone H2B as substrate. The results revealed an elevated level of AKT2 kinase in 32 of specimens (40%), including 29 cases with ductal infiltrating carcinoma, two lobular, and one mixed tumor (Fig. 1*A*). To further demonstrate AKT2 activation in breast cancer, we performed Western blot analyses of tumor lysates with phospho-Ser-473 antibody, a phosphorylation site that is critical for activation of three isoforms of Akt (17). To avoid the cross-reaction, the tumor lysates were incubated with anti-AKT2 antibody. The AKT2 immunoprecipitates were separated by SDS-PAGE and probed with phospho-Ser473 antibody. Phosphorylated AKT2 was detected only in breast tumors with elevated AKT2 kinase

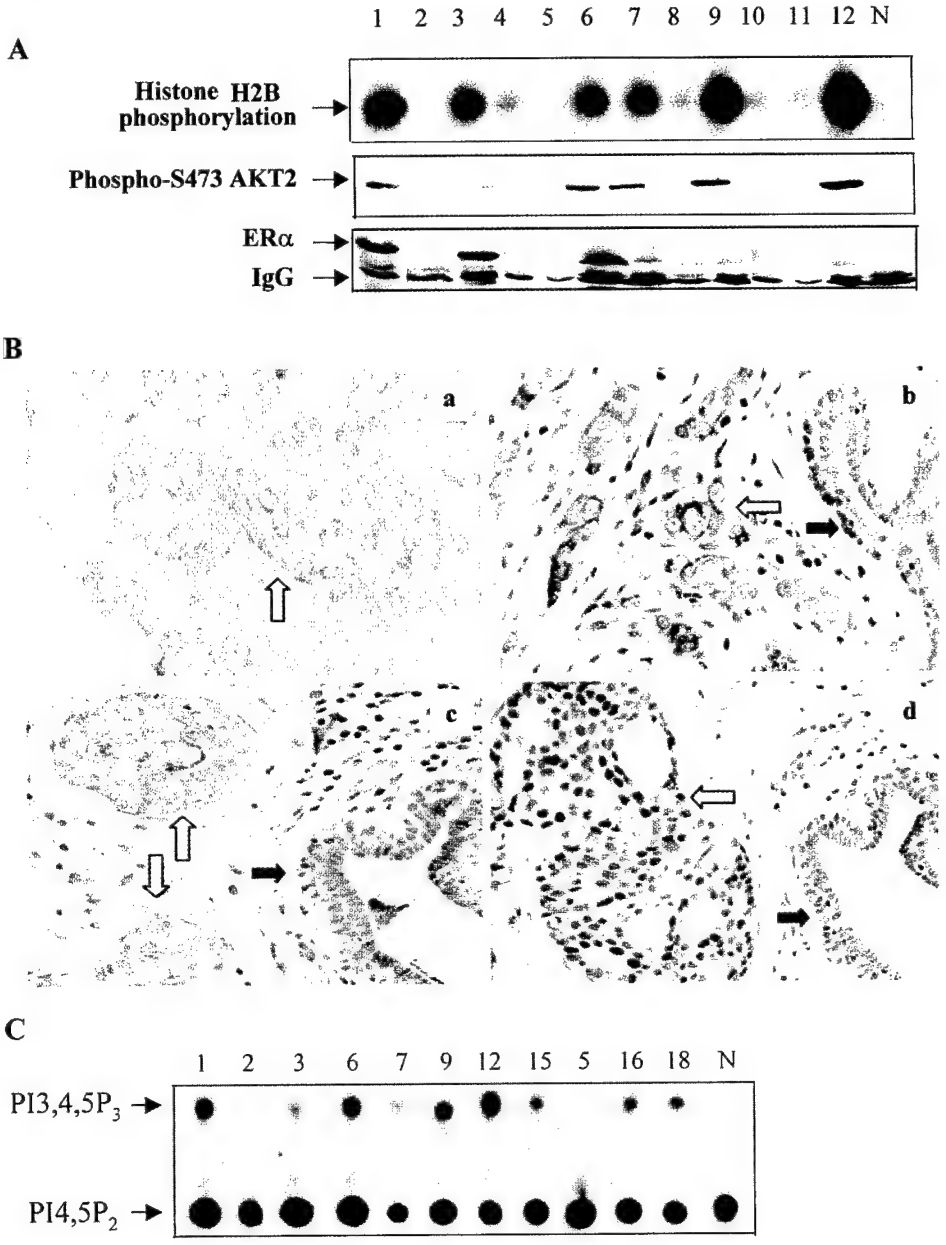

Fig. 1. Activation of AKT2 in human primary breast cancers. *A* (*top panel*), *in vitro* kinase assays of immunoprecipitated AKT2 from representative frozen breast tumor specimens. Normal mammary tissue (*N*) was used as a control. *Bottom panels*, Western blot analyses of AKT2 and ERα immunoprecipitates with anti-phospho-Ser473 Akt and anti-ERα antibodies, respectively. *B*, immunochemical staining of the paraffin sections prepared from primary breast adenocarcinomas with anti-phospho-S473 Akt (*a–c*) and anti-ERα (*d*) antibodies. Strong staining with both antibodies was observed in tumor cells (*white arrows*), whereas weak immunoreaction was detected in stromal tissue and adjacent ductal epithelium (*black arrows*). Photomicrographs *c* and *d* are the same specimen but different sections. *C*, *In vitro* PI3K assay of anti-p85 immunoprecipitates from 11 tumor and one normal specimen. The specimen numbers correspond to the same tumors shown in *A*.

activity (Fig. 1*A*). Because stromal tissues account for approximately 20–30% of the tumor specimens used in this study, we examined whether the activation of AKT2 is derived from the tumor cells or the stromal tissues by immunostaining paraffin sections with a phospho-Ser473 Akt antibody. Positive staining of tumor cells was detected in all of the 32 cases with AKT2 activation, whereas no staining was observed in normal ductal epithelial cells (Fig. 1*B*). These data suggest that activation of AKT2 is a common occurrence in human breast cancer.

Because AKT2 is a downstream target of PI3K, which is activated in colon and ovarian carcinoma (20, 22, 23), we next examined the PI3K activity in breast tumors by *in vitro* PI3K assay. Because of the fact that all of the tumors with elevated PI3K activity result in activation of Akt (20, 22–24), immunoprecipitation with a pan-p85 antibody was performed in 58 breast tumor specimens, including 32 with AKT2 activation and, as control, 26 without AKT2 activation. The ability to convert PI-4,5-P_2 to PI-3,4,5-P_3 was determined. Elevated PI3K activity was detected in all of the 32 specimens that exhibited AKT2 activation. No PI3K activation was observed in 26 specimens without AKT2 activation (Fig. 1*C*), indicating that activation of AKT2 in breast cancer predominantly results from PI3K activation. Moreover, Western blot and immunohistochemistry analyses with anti-ERα antibody revealed that 88% of the cases (28 of 32) with PI3K/AKT2 activation showed strong ERα positive (Fig. 1, *A* and *B*), whereas only 54% of the cases (14 of 26) without PI3K/AKT2 activation exhibited positive ERα, suggesting that activated PI3K/AKT2 could be involved in the regulation of ERα activity in breast cancer cells. In addition, the majority of cases with AKT2 activation are late stage (23 of 32 at stages III and IV) and poorly differentiated tumors (19 of 32), indicating that PI3K/AKT2 activation in breast cancer may be associated with tumor progression rather than initiation.

AKT2 Activates ERα-mediated Transcription in a Ligand-independent Manner. Previous studies (1, 25) have shown that MAPK is activated in breast cancer and contributes to estrogen-independent breast tumor cell growth by direct phosphorylation of ERα. Moreover, several other signal molecules, including protein kinase A, casein kinase II, $pp90^{rsk1}$, and MEKK1/p38, have been

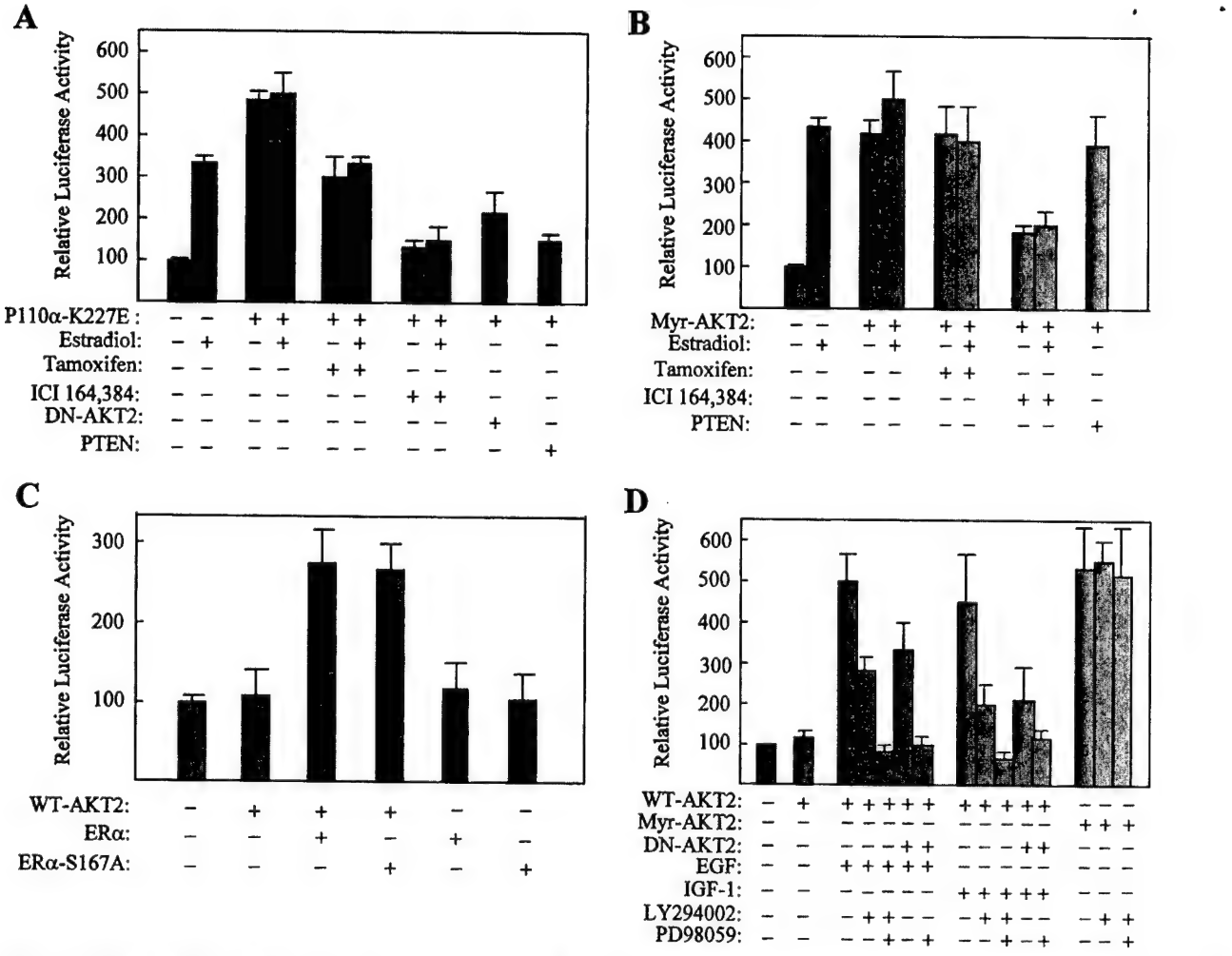

Fig. 2. AKT2 and PI3K activate ERα transcriptional activity. *A–D*, reporter assays: MCF-7 cells were transfected with ERE2-TK-LUC reporter, β-galactosidase, and indicated expression constructs. After 36 h of transfection, the cells were serum-starved overnight and treated with indicated agents. Luciferase activity was normalized to β-galactosidase activity.

shown to activate ERα-mediated transcription, possibly resulting in hormone-independent tumor cell growth (1, 8–10, 26). Because AKT2 and PI3K are frequently activated in breast cancer and the majority of cases with AKT2 activation are ERα positive, we investigated whether AKT2 and PI3K regulate ERα-mediated transcription. ERα-positive MCF7 breast cancer cells were transiently transfected with a reporter construct containing a luciferase gene regulated by two estrogen response elements (ERE2-TK-LUC) and a plasmid expressing β-galactosidase that allows the luciferase data to be normalized for transfection efficiency. In addition, the cells were transfected with expression constructs for constitutively activated p110α (p110α-K227E) subunit of PI3K, wild-type, constitutively activated, and dominant-negative AKT2 or vector alone. As shown in Fig. 2, p110α-K227E or myr-AKT2 increased ERE2-TK-LUC activity 3–4-fold in the absence of estradiol. Constitutively activated p110α-induced reporter activity was attenuated by dominant-negative mutant AKT2 (Fig. 2*A*). Tamoxifen (4-hydroxytamoxifen), an antiestrogen reagent that inhibits transcriptional activation by AF2 but not through AF1 (5), abolished estradiol-enhanced transcription but had no effects on p110α-K227E and myr-AKT2-stimulated ERα activity (Fig. 2, *A* and *B*), suggesting that PI3K/AKT2-increased ERα transcriptional activity is regulated by phosphorylation of ERα within the AF1 region and could be involved in tamoxifen resistance.

ICI 164,384, which causes rapid degradation of ERα (6, 27), completely blocked PI3K- and AKT2-induced reporter activity. PTEN, a tumor suppressor encoding a lipid phosphatase that negatively regulates PI3K, inhibited constitutively active p110-induced ERα-mediated transcription but had no effect on constitutively activated AKT2-stimulated ERα activity (Fig. 2*B*).

Moreover, we have observed that exogenous expression of ERα in ERα-positive MCF7 cells increased wild-type AKT2-induced ERE2-TK-LUC activity 2–3-fold as compared with cells transfected with wild-type AKT2 alone (Fig. 2*C*), implying that ERα might activate AKT2 kinase and subsequently enhance its own transcriptional activity (see below). Taken collectively, these data indicate that PI3K/AKT2-activated ERα-mediated transcription is estrogen-independent and that the frequently elevated level of PI3K/AKT2 kinase in primary breast cancer could relate the refractoriness of hormone therapy.

AKT2 Mediates Growth Factor-induced ERα Transcriptional Activity. A very recent study (28) showed that Akt1 mediates the estrogenic functions of EGF and IGF1. Next, we examined the possible role of AKT2 in growth factor-induced ERα activation. ERα-positive MCF7 cells were transfected with ERE2-TK-LUC and dominant-negative, wild-type, or constitutively activated AKT2 or vector alone and were treated with or without either 100 ng/ml EGF or 50 ng/ml IGF1 (Fig. 2*D*). Treatment with the growth factors resulted in an approximately 4.5-fold increase in ERα-mediated transcriptional activity. The EGF- and IGF1-induced reporter activity was partially abrogated by dominant-negative AKT2 or PI3K inhibitor LY294002 and completely blocked by the combination of PI3K and MAPK inhibitors (LY294002 and PD98059). However, the combined inhibitors had no effect on constitutively activated AKT2-induced reporter

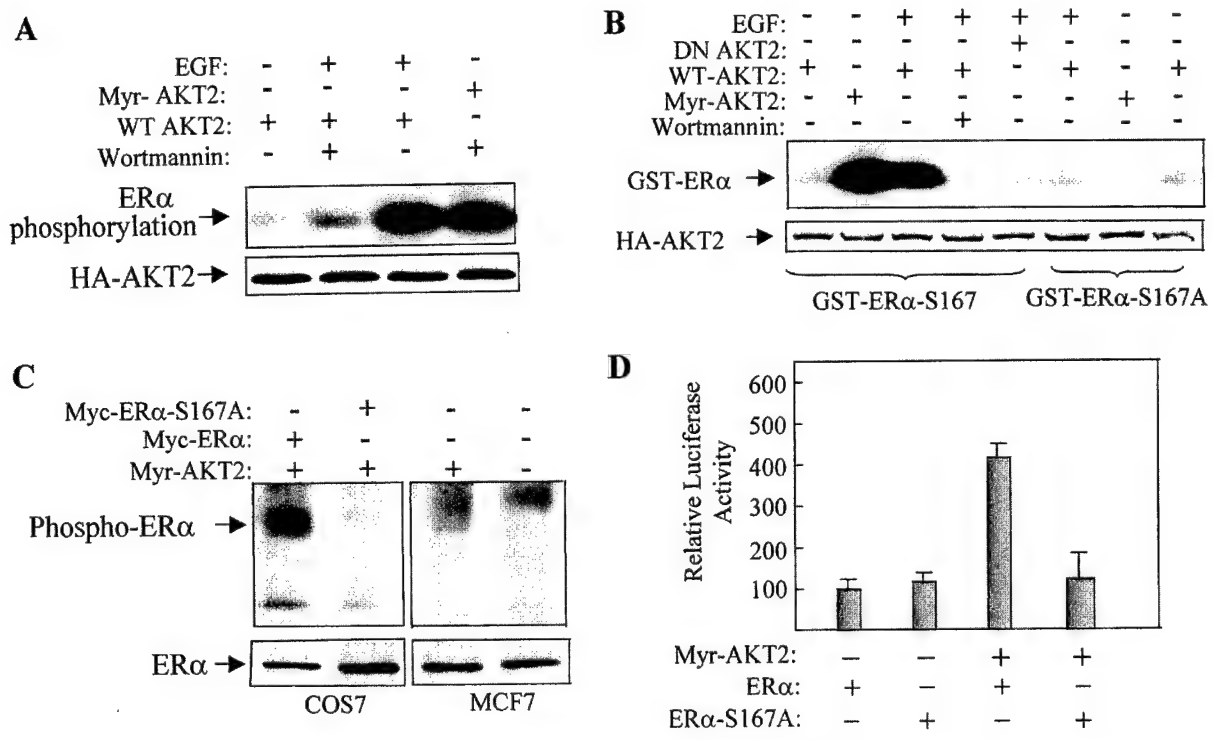

Fig. 3. AKT2 phosphorylates ERα on serine-167 *in vitro* and *in vivo*. *In vitro* AKT2 kinase assay of the immunoprecipitates from HEK293 cells transfected with indicated expression constructs. Full length of human recombinant ERα (*A*), GST-ERα-S167, and GST-ERα-S167A (*B*) were used as substrates. *C*, COS7 and MCF7 cells were transfected with indicated plasmids and incubated with $[^{32}P]P_i$ for 4 h. Immunoprecipitates were prepared with anti-myc (*left*) or anti-ERα (*right*) antibody and separated by SDS-PAGE. After transfer, the membrane was exposed to a film (*top*) and detected with anti-ERα antibody (*bottom*). *D*, AKT2 phosphorylation of serine-167 is essential for AKT2-induced ERα transcriptional activity. Luciferase reporter assay of HEK293 cells transfected with ERE2-TK-LUC, wild-type ERα, or ERα-S167A, β-galactosidase, and myr-AKT2.

activity. These results suggest that the "steroid-independent activation" of ERα by growth factors is mediated by the PI3K/AKT2 pathway, in addition to MAPK, PKA, casein kinase II, and $pp90^{rsk1}$.

AKT2 Phosphorylated Serine-167 of ERα *in Vitro* and *in Vivo*. Phosphorylation of ERα has been shown to be an important mechanism by which ERα activity is regulated. ERα is hyperphosphorylated on multiple sites in response to hormone binding and growth factor stimulation (1–4). Transcriptional activation by growth factors has been shown to require AF-1 but not AF-2 (1, 8–10). There is evidence to suggest that EGF and IGF-1 induce MAPK and $pp90^{rsk1}$/casine kinase II activity leading to phosphorylation of serine-118 and serine-167, respectively, in AF-1 region (4, 5, 8–10). To examine whether AKT2 phosphorylates ERα *in vitro*, HEK293 cells were transfected with HA-tagged wild-type and constitutively activated AKT2, and immunoprecipitation was prepared with anti-HA antibody. *In vitro* AKT2 kinase assays, using full length of human recombinant ERα as substrate, revealed that constitutively activated AKT2 and EGF-induced AKT2 strongly phosphorylated hERα. The ERα phosphorylation that was induced by EGF-stimulated AKT2 was abrogated by wortmannin (Fig. 3*A*).

To determine whether AKT2 phosphorylates ERα *in vivo*, MCF7 cells were transfected with constitutively activated AKT2 or pcDNA3 vector alone and labeled with $[^{32}P]P_i$. The cell lysates were incubated with anti-ERα antibody, and the immunoprecipitates were separated on SDS-PAGE. ERα was highly phosphorylated in constitutively activated AKT2-transfected cells but in the cells transfected with vector alone (Fig. 3*C*). These data indicate that AKT2 phosphorylated ERα both *in vitro* and *in vivo*.

Martin *et al.* (28) recently demonstrated that EGF- and IGF1-induced Akt1 potentiates the AF-1 function of ERα, possibly through the phosphorylation of serine residues. There are four serine residues (Ser-104, Ser-106, Ser-118, and Ser-167) in the AF-1 region of the receptor that are predominantly phosphorylated in response to estrogen and growth factor stimulation (1–4). We examined the ERα protein sequence and found that serine-167 (^{162}**RERLAS**^{167}) is a putative AKT2 phosphorylation site. Constructs expressing GST-fused wild-type and mutant (S167A) AF-1 region were created. *In vitro* kinase assays revealed that myr-AKT2 and EGF-stimulated AKT2 strongly phosphorylated GST-ERα-S167 but not GST-ERα-S167A mutant (Fig. 3*B*). The EGF-induced AKT2 phosphorylation of ERα is blocked by wortmannin. To examine whether AKT2 phosphorylates serine-167 *in vivo*, COS7 cells were transfected with myc-tagged wild-type and mutant (S167A) human ERα expression constructs together with constitutively activated AKT2. After 36 h of transfection, the cells were incubated with $[^{32}P]P_i$ and immunoprecipitated with anti-myc antibody. As demonstrated in Fig. 3*C*, constitutively active AKT2 phosphorylated wild-type ERα but not the ERα-S167A mutant *in vivo*, suggesting that serine-167 of ERα is a physiological substrate for AKT2.

Previous studies (29) showed that serine-167 is important for ERα transcriptional activity. To further examine whether AKT2-activated ERα transcriptional activity depends upon phosphorylation of serine-167, reporter assays were carried out in HEK293 cells transfected with ERE2-TK-LUC, constitutively activated AKT2, and ERα-S167A or wild-type ERα. Fig. 3*D* shows that ERα-S167A had no ability to mediate constitutively activated AKT2-induced ERE2-TK-LUC reporter activity, indicating that AKT2 regulates ERα-mediated transcription through phosphorylation of serine-167.

ERα Binds To and Activates PI3K/AKT2 in Epithelial Cells via a Ligand-independent Mechanism. Recent studies (30, 31) demonstrated that ERα binds to the p85α regulatory subunit of PI3K after estradiol treatment, leading to the activation of PI3K/Akt and endo-

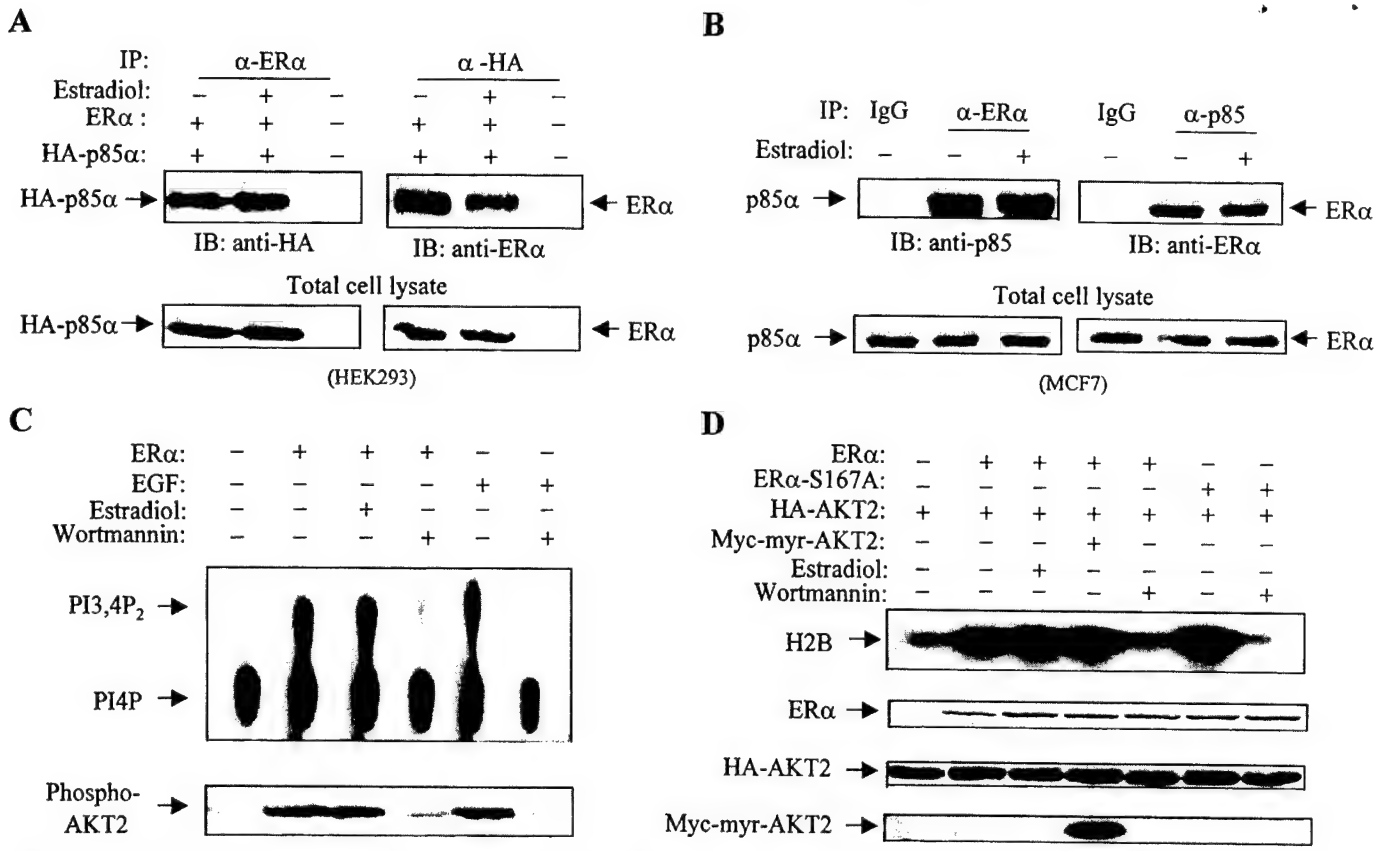

Fig. 4. ERα interacts with p85α and activates the PI3K/AKT2 pathway in human epithelial cells. Coimmunoprecipitation of ERα and p85α in (*A*) HEK293 cells cotransfected with HA-p85α/ERα and in (*B*) nontransfected MCF7 cells. *Top*, coimmunoprecipitation; *bottom*, Western blot of total cell lysates. *C*, *in vitro* PI3K assay (*top*) of HEK293 cells transfected and treated with indicated plasmid and agents. *Bottom*, Western blotting analysis of AKT2 immunoprecipitates with phospho-S473 Akt antibody. *D*, *in vitro* kinase assay (*top*) of HA-AKT2 immunoprecipitates prepared from HEK293 cells transfected with indicated expression constructs, using histone H2B as a substrate. *Panels 2–4*, Western blots of transfected HEK293 cell lysates detected with anti-ERα, anti-HA, or anti-myc antibody.

thelial nitric oxide synthase in endothelial cells. In the absence of estradiol, ERα failed to bind and activate PI3K, indicating that ERα-associated PI3K in endothelial cells is estrogen-dependent (30). Next, we examined whether ERα binds to and activates PI3K/AKT2 in epithelial cells. ERα/HA-p85α-transfected HEK293 and nontransfected ERα-positive MCF7 cells were immunoprecipitated with anti-ERα and detected with anti-HA or anti-p85α antibody or *vice versa*. As shown in Fig. 4*A* and *B*, ERα constitutively associated with p85α, and this interaction was unaffected by estradiol treatment. In addition, *in vitro* PI3K assays revealed that expression of ERα in HEK 293 cells significantly induced PI3K activity in the absence or presence of estradiol (Fig. 4*C*). These data suggest that ERα binding to and activating PI3K is ligand-independent in epithelial cells.

Next, we examined whether ERα activates AKT2 and whether this activation is dependent on AKT2 phosphorylation. ERα-negative HEK293 cells were transfected with ERα or ERα-S167A, together with HA-AKT2. *In vitro* AKT2 kinase assays revealed that ERα significantly activates AKT2 in the absence of estradiol. Additional estradiol treatment did not further enhance ERα-induced AKT2 activation. The PI3K inhibitor, wortmannin, completely abolished the activation. Interestingly, ERα-S167A activated AKT2 at a similar level to that of wild-type ERα. Coexpression of myc-tagged constitutively active AKT2 (Myc-myr-AKT2) and ERα had the same effect on wild-type AKT2 activation as that of expression of ERα alone (Fig. 4*D*). These results indicate that activation of AKT2 by ERα is through PI3K and independent of ERα phosphorylation by PI3K/AKT2.

In summary, we demonstrate in this study that AKT2 and PI3K are frequently activated in primary human breast carcinoma. The PI3K/AKT2 pathway regulates ERα transcriptional activity by phosphorylation of serine-167 *in vitro* and *in vivo*, and ERα activates PI3K/AKT2 kinase by binding to p85α in a ligand-independent manner in epithelial cells. This study suggests that the PI3K/AKT2 pathway may play a pivotal role in estrogen-independent breast cancer cell growth and tamoxifen-resistance; therefore, it could represent an important therapeutic target in human breast cancer.

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Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer

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We previously demonstrated that AKT2, a member of protein kinase B family, is activated by a number of growth factors via Ras and PI 3-kinase signaling pathways. Here, we report the frequent activation of AKT2 in human primary ovarian cancer and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase (PI 3-kinase)/Akt pathway. *In vitro* AKT2 kinase assay analyses in 91 ovarian cancer specimens revealed elevated levels of AKT2 activity (>3-fold) in 33 cases (36.3%). The majority of tumors displaying activated AKT2 were high grade and stages III and IV. Immunostaining and Western blot analyses using a phospho-ser-473 Akt antibody that detects the activated form of AKT2 (AKT2 phosphorylated at serine-474) confirmed the frequent activation of AKT2 in ovarian cancer specimens. Phosphorylated AKT2 in tumor specimens localized to the cell membrane and cytoplasm but not the nucleus. To address the mechanism of AKT2 activation, we measured *in vitro* PI 3-kinase activity in 43 ovarian cancer specimens, including the 33 cases displaying elevated AKT2 activation. High levels of PI 3-kinase activity were observed in 20 cases, 15 of which also exhibited AKT2 activation. The remaining five cases displayed elevated AKT1 activation. Among the cases with elevated AKT2, but not PI 3-kinase activity (18 cases), three showed down-regulation of PTEN protein expression. Inhibition of PI 3-kinase/AKT2 by wortmannin or LY294002 induces apoptosis in ovarian cancer cells exhibiting activation of the PI 3-kinase/AKT2 pathway. These findings demonstrate for the first time that activation of AKT2 is a common occurrence in human ovarian cancer and that PI 3-kinase/Akt pathway may be an important target for ovarian cancer intervention. *Oncogene* (2000) **19,** 2324–2330.

Keywords: Akt; PI 3-kinase; signal transduction; ovarian cancer

Akt/PKB represents a subfamily of the serine/threonine protein kinases (Bellacosa *et al.*, 1991; Jones *et al.*, 1991a, b; Cheng *et al.*, 1992; Konishi *et al.*, 1995; Nakatani *et al.*, 1999). Three members of this family, AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ have been identified. Akt is activated by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress (Franke *et al.*, 1995; Burgering *et al.*, 1995; Shaw *et al.*, 1998; Liu *et al.*, 1998). Activation of Akt and AKT2 by growth factor is mediated by PI 3-kinase (Franke *et al.*, 1995; Meier *et al.*, 1997; Liu *et al.*, 1998). Active Ras and Src have also shown to activate Akt and AKT2 and this activation is blocked by wortmannin, a PI 3-kinase inhibitor, indicating that Ras and Src also mediate the activation of Akt and AKT2 and are located upstream of PI 3-kinase (Datta *et al.*, 1996; Liu *et al.*, 1998). Several lines of evidence suggest that PI 3-kinase regulates Akt activation through the following mechanism: the product of PI 3-kinase, phosphatidylinositol-3,4,5-trisphosphate, binds to the pleckstrin homology (PH) domain of Akt after growth factor stimulation, resulting in recruitment of Akt to the cell membrane. A conformational change of Akt follows, which allows residues Thr-308 and Ser-473 to be phosphorylated by upstream kinases, PDK-1 and PDK2 or ILK, respectively (Alessi and Cohen, 1998; Delcommenne *et al.*, 1998). Several downstream targets of Akt, each of which contains the Akt phosphorylation consensus sequence R-X-R-X-X-S/T-F/L, have been identified (Alessi and Cohen, 1998), pointing to the possible mechanisms by which Akt promotes cell survival and blocks apoptosis. One such target is GSK3. Akt phosphorylates GSK3 and leads to inactivation of GSK3, accumulation of β-catenin, and activation of *Myc* transcription. There is also evidences that Akt phosphorylates the proapoptotic proteins BAD, caspase-9 and transcription factor FKHRL1, resulting in reduced binding of BAD to Bcl-X_L and inhibition of caspase-9 protease activity and Fas ligand transcription (Datta *et al.*, 1997; del Peso *et al.*, 1997; Cardone *et al.*, 1998; Brunet *et al.*, 1999). Recent studies demonstrated that PTEN/MMAC1 tumor suppressor and SHIP, tyrosine and inositol phosphatases, dephosphorylate phosphatidylinositol-3,4,5-triphos-phate, thus, inhibiting the PI 3-kinase/Akt signaling pathway (Stambolic *et al.*, 1998; Aman *et al.*, 1998).

Several members within the PI 3-kinase/Akt pathway, including PI 3-kinase, PTEN, AKT2, and β-catenin, have been implicated in human neoplasms (Alessi and Cohen, 1998). Overexpression of p85, the regulatory subunit, or p110, the catalytic subunit, of PI 3-kinase is able to transform cells (Chang *et al.*, 1997; Jimenez *et al.*, 1998). Alterations of PI 3-kinase have also been detected in a number of human malignancies (Shayesteh *et al.*, 1999; Phillips *et al.*, 1998). Mutation and/or down regulation of the *Pten* are frequently observed in endometrial carcinoma, glioblastoma, breast cancer and prostate carcinoma (Stambolic *et*

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al., 1998). We and others have previously demonstrated alterations of *AKT2* at DNA, mRNA and/or protein level in several types of human malignancy (Cheng *et al.*, 1992, 1996; Bellacosa *et al.*, 1995; Ruggeri *et al.*, 1998). In particular, amplification/overexpression of *AKT2* has been detected in 10–20% of ovarian carcinomas and pancreatic cancers. However, increased activation of AKT2 in human tumors has not been demonstrated previously.

In this report, we show that kinase activity of AKT2 is frequently elevated in human primary ovarian tumor. *In vitro* AKT2 kinase assays were performed in 91 primary human ovarian tumors, including 34 serous cystadenocarcinomas, four mucinous cystadenocarcinomas, 25 papillary serous adenocarcinomas, 10 endometrioid adenocarcinomas, two borderline tumors, five granulosa cell tumors and 11 other rare types of tumors including adenosarcoma, thecoma, fibroma, and mesodermal mixed tumor. Lysates from tumor specimens containing equivalent amounts of protein were precleared and incubated with anti-AKT2 antibody, which specifically reacts with AKT2, in the presence of protein-A : protein-G beads. The immunoprecipitates were subjected to *in vitro* kinase assay using Histone H2B as the substrate. *In vitro* kinase assays were carried out three times for each specimen. Average reading of the kinase activity threefold higher than that in normal ovarian tissue was considered elevated AKT2 activity. The results revealed an elevated level of AKT2 kinase in 33 specimens (36.3%), including 15 serous cystadenocarcinomas (44%), 14 papillary serous adenocarcinomas (56%) and four other types of tumor including one adenosarcoma, two malignant clear cell tumors, and one mixed mullerian tumor. Examples of AKT2 kinase activity in ovarian cancer are shown in Figure 1. Interestingly, the majority of cases exhibiting activation of AKT2 were serous adenocarcinomas (29/33). None of endometrioid, borderline, mucinous, and granulosa cell tumors exhibited elevated AKT2 kinase activity.

To examine whether variations in AKT2 protein expression level contributes to the level of AKT2 activity observed in ovarian tumor specimens, we analysed its expression by Western blot analyses using a specific anti-AKT2 antibody. A high level of AKT2 protein was detected in 42 of 91 cases (46%). The 33 cases that exhibited elevated AKT2 activity displayed variable levels of AKT2 protein, 25 of which expressed AKT2 at high or moderate levels (Figure 1 and Table 1). These results were also confirmed by immunohistochemical staining of the tumor tissue section (data not shown).

Figure 1 Elevated level of AKT2 kinase activity in ovarian cancer. Top panel: *In vitro* kinase assays of AKT2 immunoprecipitated from 15 frozen representative tumor specimens. Frozen tissues from ovarian carcinoma was mechanically smashed in liquid nitrogen and lysed by a Tissue Tearor in a lysis buffer (Liu *et al.*, 1998). Lysates were incubated with anti-AKT2 antibody in the presence of protein A-protein G (2:1) agarose beads for 2 h at 4°C. Following extensive wash, immunoprecipitates were subjected to *in vitro* kinase assay (Liu *et al.*, 1998). Histone H_2B was used as the exogenous substrate. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Phosphorimager. AKT2 is activated in cases T2, T5, T7, T10, T13, and T15. Middle panel: Western blot analysis of AKT2 expression in ovarian carcinoma. The blot was reprobed with anti-β-actin antibody (Bottom panel)

Previous studies demonstrated that phosphorylation of Thr-308 and Ser-473 of Akt1 is required for its full activation. Upon stimulation of PI 3-kinase by growth factors, Akt1 becomes phosphorylated at these two residues. It has been shown that AKT2 is activated by phosphorylation of the equivalent residues (Thr-309 and Ser-474) (Alessi and Cohen, 1998). The homology of Akt1 to AKT2 and AKT3 is 90.4% and 87.8% at the amino acid level, respectively (Cheng *et al.*, 1992; Nakatani *et al.*, 1999), and the sequence of the serine-473 phosphorylation site of Akt1 is highly conserved in AKT2 and AKT3 (Figure 2a).

Anti-phospho-Ser473 Akt antibody has widely been used to identify Akt1 activation. To determine whether such an antibody can be used to detect AKT2 activation in ovarian cancer specimens by Western blotting and immunostaining, we tested whether a phospho-Ser473 Akt antibody reacted with phosphorylated AKT2. HA-AKT2 expression construct was transfected to COS-7 cells. After serum-starvation overnight and IGF-1 stimulation for 10 min, HA-AKT2 was immunoprecipitated with anti-HA monoclonal antibody. The immunoprecipitates were separated by SDS–PAGE and probed with phospho-Ser473 Akt antibody. The results showed that the antibody against phospho-Ser473 Akt strongly reacted with phospho-Ser 474 of AKT2 (Figure 2b).

We next examined AKT2 phosphorylation by Western blot analyses of AKT2 immunoprecipitates with phospho-Ser473 Akt antibody in 43 ovarian tumor specimens including the 33 cases with and 10 cases without elevated AKT2 kinase activity. A sheep anti-AKT2 antibody was used to incubate with equal amount of protein from ovarian tumor lysates in the presence of protein-A and protein-G agarose. After extensive wash with the lysis buffer, the AKT2 immunoprecipitates were separated by SDS–PAGE and the blots were probed with the rabbit polyclonal phospho-Ser473 Akt antibody (New England Biolab). Phosphorylated AKT2 was detected only in ovarian tumors with elevated AKT2 kinase activity; for example, see data from tumors T2, 5, 7, 10 and 13, which exhibit high AKT2 kinase activity, shown in Figure 2c. These data confirm the results obtained from *in vitro* AKT2 kinase assay and indicate that AKT2 activity is regulated by phosphorylation at Ser474 in human primary tumors.

Table 1 AKT2 activation and clinical stage

		AKT2 kinase activity		*AKT2 protein level*	
Stage	n	*Normal*	*High*	*Low*	*High/moderate*
I	7	7	0	5	2
II	11	11	0	6	5
IIIA	5	5	0	1	4
IIIC	53	27	26	31	22
IV	15	8	7	6	9

Due to the fact that stromal tissues approximately account for 20~30% of the tumor specimens used in this study, we examined whether activation of AKT2 is derived from tumor cells or stromal tissues by immunostaining the tumor paraffin sections with phospho-Ser473 Akt antibody. We first tested if the phospho-Ser473 Akt antibody is capable of recognizing phosphorylated AKT2 by immunohistochemistry. Cell paraffin blocks were prepared from serum-starved and serum-starved/EGF-stimulated OVCAR-3 cells that overexpress AKT2 (Cheng *et al.*, 1992). Phospho-Ser473 antibody was used to detect phosphorylated AKT2 in the section from these blocks by immunohistochemical means. Strong positive staining was observed in EGF-stimulated but not serum-starved OVCAR-3 cells (panel 1~2 in Figure 3a). Phosphorylation status of AKT2 in these cells was confirmed by Western blot analysis with phospho-S473 Akt antibody (lanes 1 and 2 in Figure 3b).

Immunostaining of the tumor paraffin sections with phospho-Ser473 antibody was performed in 43 ovarian cancer specimens including 33 cases exhibiting elevated AKT2 kinase activity and 10 cases without AKT2 activation. Positive staining of tumor cells was observed in 38 cases including 33 with and five without AKT2 activation detected by *in vitro* kinase assay and Western blot analyses. The fact that phospho-Ser473 Akt antibody recognizes Akt1, AKT2 and possible AKT3 suggests that activation of AKT1 or AKT3 present in these five cases, which may be due to different expression levels of three isoforms of Akt. Immunohistochemical staining of paraffin tissue sections with anti-AKT1 and -AKT2 antibodies revealed that AKT1 but not AKT2 is highly expressed in these five specimens (Figure 3c). Moreover, *in vitro* kinase assay showed that AKT1 kinase activity is elevated in these specimens (Figure 3d, and also see case 8 in Figures 1 and 4). Unfortunately, no good AKT3 antibody is commercially available at present time, therefore, we could not determine if AKT3 protein is altered in these specimens. Strikingly, phosphorylated Akt localizes to cytoplasm and cell membrane. Even in EGF-stimulated OVCAR-3 cells, activated Akt stays in cytoplasm and cell membrane. No nuclear staining was observed (panels 2 and 4 of Figure 3a).

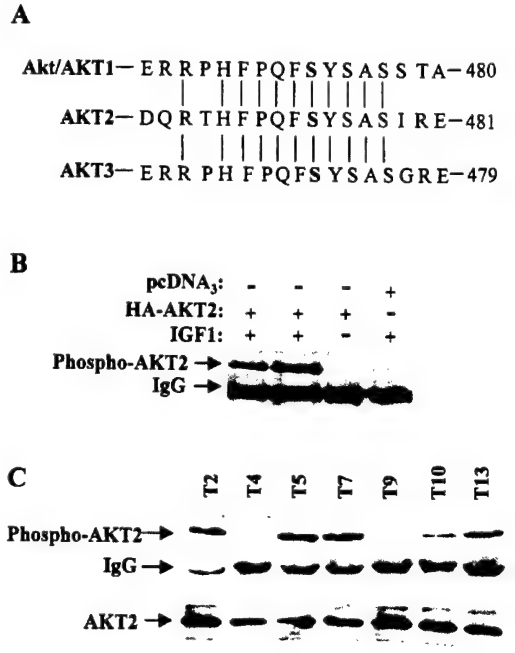

Figure 2 Phospho-Akt-Ser473 antibody recognizes phosphorylation form of AKT2 that is detected in ovarian cancer. (**a**) Comparison of C-terminal amino acid sequence of AKT1 and AKT2 and AKT3. Phosphorylation of serine-473 (AKT1) is well conserved in AKT2 (Ser-474) and AKT3 (Ser-472). (**b**) Western blot analysis of HA-AKT2 immunoprecipitates from COS-7 cells transfected with $pcDNA_3$-HA-AKT2 or $pcDNA_3$ vector alone. After transfection, the cells were serum-starved overnight and stimulated with IGF-1 for 10 min (lanes 1 and 2 from left) prior to harvesting cells. Immunoprecipitation was carried out with anti-HA monoclonal antibody and separated by SDS–PAGE. The blot was detected with Phospho-Akt-Ser473 antibody. (**c**) Western blot analysis of phosphorylation of AKT2 in ovarian cancer specimens. The tumor lysates were incubated with anti-AKT2 antibody. The resulting immunoprecipitates were separated by SDS–PAGE and detected with polyclonal phospho-Ser473 Akt antibody (upper panel) or polyclonal anti-AKT2 antibody (lower panel)

One mechanism that could result in an increased activation of AKT2 is an up-regulation of PI 3-kinase. PI 3-kinase is a heterodimer composed of a p85-regulatory and a p110-catalytic subunit. Three isoforms of p85 and p110 have been cloned, namely p85α, p85β, p85γ, p110α, p110β and p100γ. Recent studies demonstrated transforming activity of p110 and p85 and frequent activation PI 3-kinase in colon and ovarian carcinoma cell lines (Chang *et al.*, 1997; Jimenez *et al.*, 1998; Shayesteh *et al.*, 1999; Phillips *et al.*, 1998). To examine PI 3-kinase activity in tumor tissues, immunoprecipitation with a pan-p85 specific antibody was performed in 43 ovarian cancer cases, including 33 with and 10 without AKT2 activation (Klippel *et al.*, 1993). Following extensive wash, the immunoprecipitates were subjected to *in vitro* PI 3-kinase assay using L-α-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P_2) or L-α-phosphatidylinositol-4-phosphate (PI-4-P_1) as substrates. The conversion of PI-4,5-P_2 to PI-3,4,5-P_3 or PI-4-P_1 to PI-3,4-P_2 was determined by autoradiography and quantitated by Phosphorimager. Elevated PI 3-kinase activity was detected in 20 ovarian cancer specimens, 15 of which exhibited AKT2 activation and the other five displayed AKT1 activation (Figures 3 and 4). Since p110α has been shown to be over-expressed and activated in ovarian cancer cell lines (Shayesteh *et al.*, 1999), we examined whether increased levels of activity observed in our primary ovarian tumor specimens resulted from increased levels of p110 expression. PI 3-kinase was immunoprecipitated with an anti-p85 antibody from equal amounts of tumor extract protein. The immunoprecipitates were then separated on a SDS–PAGE, transferred to a membrane and probed with a specific anti-p110α antibody. Figure 4 shows a correlation between p110α expression levels and PI 3-kinase activity in tumor samples. Quantitative analysis by a BioImager (Genomic Solutions Inc.) revealed that the levels of p110α expression in the tumors with PI 3-kinase activation are 2~4-folds higher than that in the specimens showing no elevated PI 3-kinase, except T12 that exhibited high level of p110α protein but no significant PI 3-kinase activation. These data indicate that increases of PI 3-kinase activity contribute to AKT activation in human primary ovarian carcinoma.

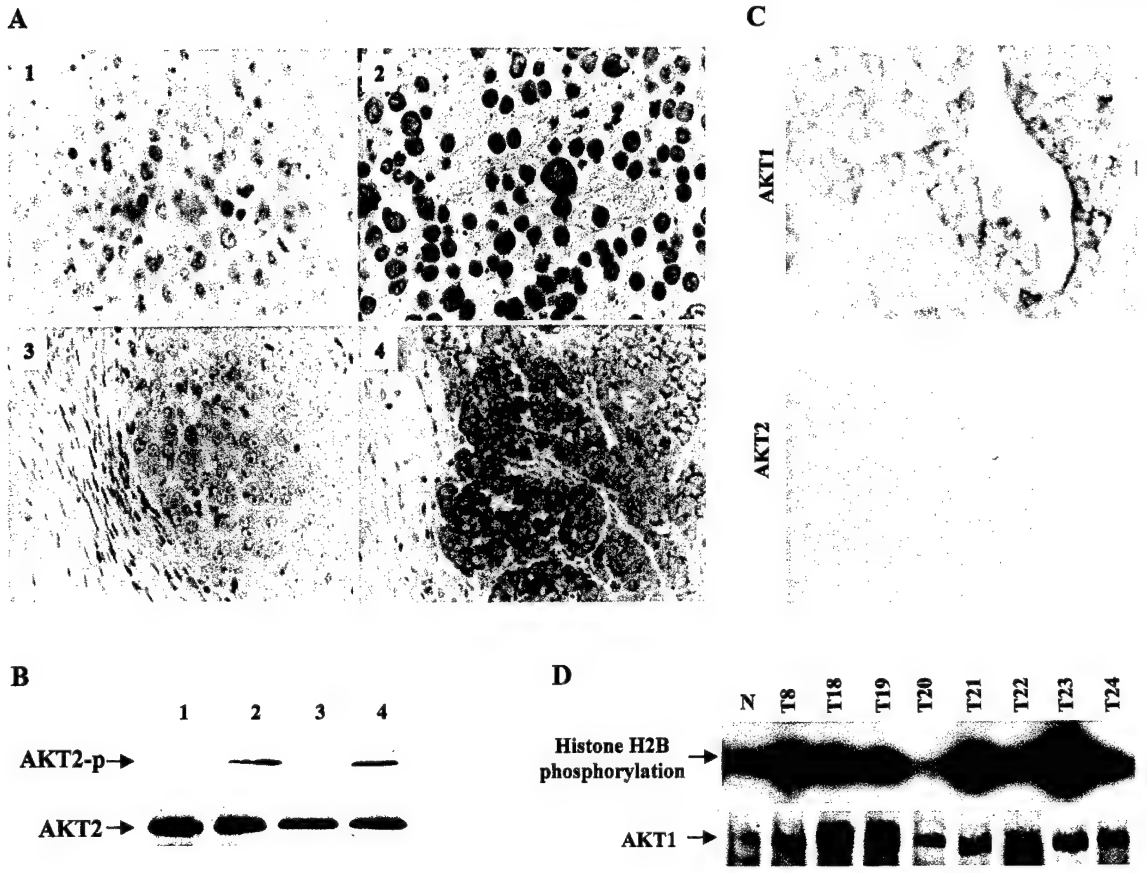

Figure 3 Phosphorylated Akt detected by immunostaining localizes to cell membrane and cytoplasm of ovarian tumor cells. The paraffin sections were subjected to antigen retrieval by boiling in a microwave and then incubated in a blocking solution and an avidin/biotin blocking kit (Vector). The primary antibody to phospho-S473 Akt (Upstate Biotechnology) was applied at a dilution of 1:200. After incubation, the slides were treated with biotinylated rabbit anti-goat immunoglobulin and streptavidin and biotinylated alkaline phosphatase. **(a)** Sections of paraffin blocks prepared from serum-starved (1), EGF-stimulated OVCAR3 cells (2) and 2 primary ovarian carcinoma specimens (3~4) were stained with phospho-Ser473 antibody. No staining was observed in serum starved OVCAR3 cells and a specimen without elevated AKT2 kinase activity (1 and 3), whereas strong reaction with phospho-Ser473 antibody is seen in EGF-stimulated OVCAR3 cells and a tumor specimen exhibiting AKT2 activation. **(b)** Western blot analysis of the AKT2 immunoprecipitates from the cells and the tissues that are used for immunostaining in Figure 3a. The filter was detected with phospho-Ser473 antibody (upper panel) and anti-AKT2 antibody (lower panel). **(c)** Immunohistochemical staining of an ovarian cancer specimen with anti-AKT1, and -AKT2 antibodies. **(d)** *In vitro* kinase assay (upper panel) and Western blot (bottom panel) of AKT1 immunoprecipitates from frozen tumor specimens. Results represent three independent experiments. AKT1 is activated in cases T8, T18, T19, T21, and T23

Another mechanism shown to result in activation of Akt is inactivation or loss of expression of PTEN. Inactivating mutations of the *Pten* tumor suppressor gene, on chromosome 10q23, have been described in prostate, endometrial and ovarian endometrioid carcinomas (Obata *et al*., 1998). Previous studies have also observed down-regulation of PTEN protein in prostate cancer (Wu *et al*., 1998; McMenamin *et al*., 1999). Therefore, we examined whether down-regulation of PTEN is associated with AKT2 activation in ovarian cancer. We analysed PTEN expression in 18 ovarian cancer specimens displaying elevated AKT2 activity by Western blot. A large reduction in PTEN protein expression was observed in three cases, two of which were serous cystadenocarcinomas and one was a papillary serous adenocarcinoma (Figure 5). In these cases, we did not detect either mutations or low mRNA level of the *Pten* gene (data not shown). Interestingly, we failed to observe elevated AKT2 kinase activity in 10 endometrioid adenocarcinomas examined, even though PTEN mutations have most frequently been detected in this type of ovarian tumor. These results suggest that down-regulation of PTEN protein expression, either by translational or post-translational changes, may be a factor in elevating AKT2 activation in non-endometrioid ovarian cancer.

We next examined the relationships between activation of AKT2 and tumor stage and grade. The results are presented in Tables 1 and 2. High levels of AKT2 activity were seen in stages III and IV (33/68, 48.5%) but not stages I and II. The incidence of AKT2 activation increased with increasing grade. High levels of AKT2 kinase activity were detected in 52.9% of grades III and IV tumors, whereas activation of AKT2 was observed in only 15% of grades I and II tumors. These data indicate that activation of AKT2 in ovarian carcinoma is associated with late stage and high-grade tumors, and suggest an increase in the activation of AKT2 kinase as ovarian cancer progresses to a more aggressive phenotype.

It has been shown that Akt induces cell survival and suppresses the apoptotic death of a number of cell types induced by a variety of stimuli, including growth factor

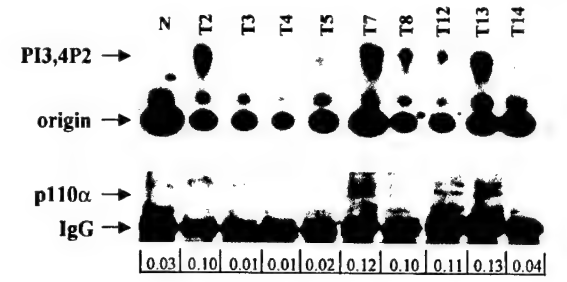

Figure 4 Activation of PI 3-kinase in human ovarian cancer specimens. *In vitro* PI 3-kinase assay of the anti-p85 immunoprecipitates from 12 frozen ovarian cancer specimens. The tumor tissue lysates were immunoprecipitated with pan-p85 antibody (Santa Cruz). Following wash, the presence of PI 3-kinase activity in immunoprecipitates was determined by incubating the beads with reaction buffer. Phospholipids were extracted and separated by thin-layer chromatography as previously described (Jiang *et al*., 2000). The conversion of PI-4,5-P_2 to PI 3-phosphate and PI-4-PI to PI-3,4-P_2 was determined by autoradiography and quantitated by using a Phosphorimager. Elevated levels of PI 3-kinase activity (top panel) and p110α protein (middle panel) are detected in cases 2, 7, 8, 12, and 13. The expression levels of p110α are quantified by a BioImager. The density of each band is indicated at the bottom

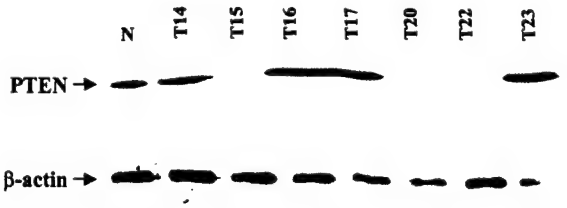

Figure 5 Down regulation of PTEN in human ovarian carcinoma. Western blot analyses of the tumor tissue lysates with anti PTEN (upper) and β-actin (bottom) antibody. No detectable level of PTEN was observed in three cases

Table 2 AKT2 activation and grade

		AKT2 kinase activity	
Grade	n	*Normal*	*High*
1	21	17	4
2	19	17	2
3	49	24	25
4	2	0	2

withdrawal, cell-cycle discordance and loss of cell adhesion. To assess the influence of PI 3-kinase/AKT activation on the cell growth of human ovarian cancer cells, we have performed *in vitro* kinase assay and observed activation of AKT2 and PI 3-kinase in 2 of 5 ovarian cancer cell lines (Figure 6a). If PI 3-kinase/Akt pathway is crucial for survival in such ovarian tumor cells, blocking the activity of PI 3-kinase/Akt is expected to inhibit the cell growth and/or induce apoptosis. To test this hypothesis, three ovarian cancer cell lines with or without PI 3-kinase/AKT2 activation were treated with PI 3-kinase inhibitors, wortmannin (200 nM) or LY294002 (40 μM), or vehicle (DMSO), for 12 h in a medium containing 1% fetal calf serum. Those cell lines exhibiting elevated levels of PI 3-kinase and AKT2 activity underwent apoptosis after treatment with wortmannin or LY294002, whereas no apoptosis was detected in the cell line (e.g., A2780) without PI 3-kinase/AKT2 activation (Figure 6).

In this study, we have demonstrated frequent activation of AKT2 kinase in human primary ovarian cancer by *in vitro* kinase assay, Western blot and immunohistochemical staining. Phosphorylated AKT2 has a cell membrane and cytoplasmic but not nuclear localization. Increased PI 3-kinase activation is observed in the majority of cases displaying AKT2 activation. High levels of AKT2 protein expression are also frequently detected in ovarian cancer specimens. Down regulation of PTEN was observed in non-endometrioid ovarian tumors, which may contribute to AKT2 activation. Moreover, activation of AKT2 is associated with high grade and late stage ovarian cancer and direct inhibition of PI 3-kinase/Akt pathway induces apoptosis in ovarian cancer cell lines exhibiting activation of PI 3-kinase and AKT2.

Previous studies showed that alteration of oncogene could occur at DNA, mRNA, protein, or enzymatic level. An extensive review of the topic indicates that the frequency of oncogene amplification in primary tumors is about 5–38%, depending on tumor type, stage, and grade as well as the individual gene (Brison, 1993). The incidence of overexpression of some oncogenes in certain tumors is even higher due to the mechanism of enhanced transcription or translation, and mRNA or protein stabilization (Brison, 1993; Devilee and Cornelisse, 1994; Berns *et al*., 1995). We and others have previously shown alterations of *AKT2* at DNA and mRNA levels in ~12% and ~25% of ovarian carcinoma, respectively (Cheng *et al*., 1992; Bellacosa *et al*., 1995). In this study, we demonstrate activation of AKT2 kinase in 36.3% of primary ovarian tumors with highest frequency (49%) in serous adenocarcinomas. Moreover, we have also observed overexpression of AKT2 protein in 46% of ovarian cancer specimens examined. These data, as compared to *AKT2* alteration at DNA and RNA levels, indicate that activation and overexpression of AKT2 protein by aberrant translational regulation and posttranslational modification of AKT2 is a more common occurrence in human ovarian cancer. The fact that overexpression of wild type AKT2 in NIH3T3 cells results in malignant transformation (Cheng *et al*., 1997) suggests that activation and overexpression of AKT2 in human ovarian carcinoma could play an important role in the development of this malignancy.

Several studies demonstrated that ectopically expressed Akt1 and AKT2, following growth factor stimulation, initially locates to cell membrane and then translocates to the nucleus (Andjelkovic *et al*., 1997; Meier *et al*., 1997). This is thought to allow Akt mediated phosphorylation of nuclear transcription factors, such as Forkhead proteins and CREB (Brunet *et al*., 1999; Kops *et al*., 1999; Du and Montminy, 1998). In contrast, in primary ovarian tumor specimens examined, we found that phosphorylated AKT2 localizes to cell membrane and cytoplasm but not the nucleus. Moreover, activated endogenous AKT2 in AKT2-overexpressing OVCAR cells was also localized to cell membrane and cytoplasm only. These conflicting observations may be due to the fact that ectopic overexpression of Akt affects its subcellular localization.

We and others have previously documented that AKT2 is a downstream target of PI 3-kinase and is activated by a number of growth factors (Meier *et al*.,

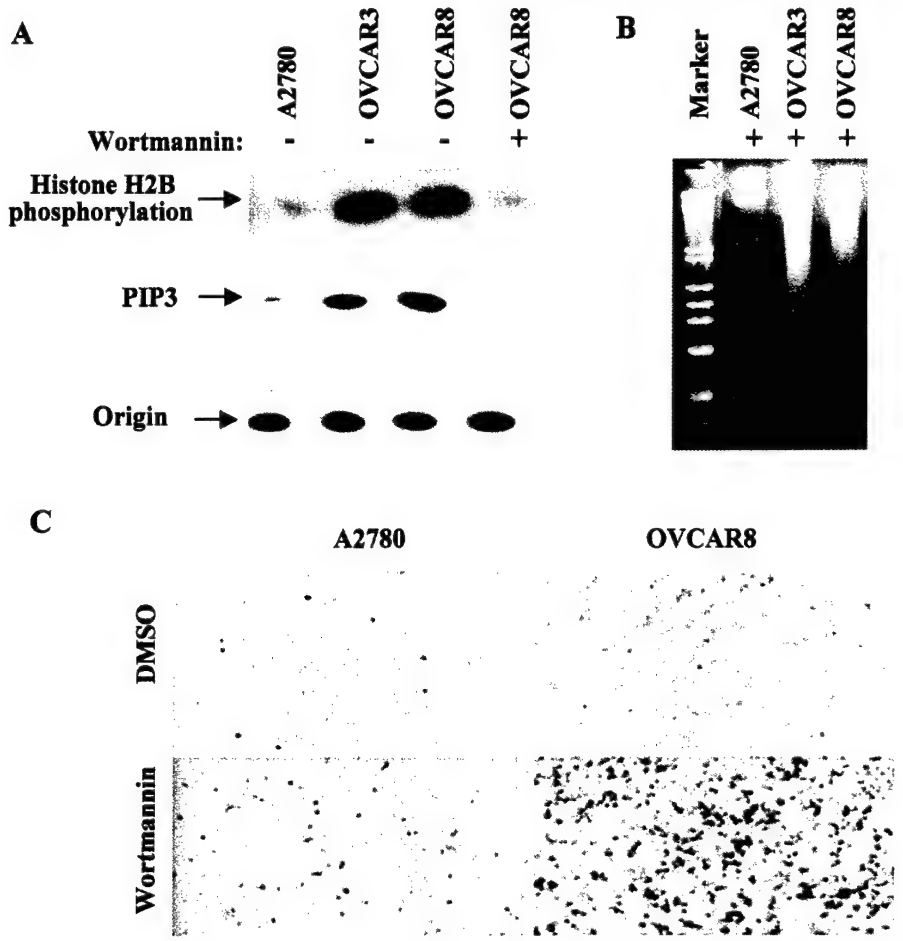

Figure 6 Inhibition of PI 3-kinase/AKT2 activity induces apoptosis in PI 3-kinase/AKT2-activating ovarian cancer cell lines. (**a**) *In vitro* Akt and PI 3-kinase assay of the immunoprecipitates from A2780, OVCAR3 and OVCAR8 cells. AKT2 (upper panel) and PI 3-kinase (bottom panel) are activated in OVCAR3 and OVCAR8 cells. (**b**) and (**c**) DNA fragmentation and Tunel assay showing that Wortmannin blocks activity of PI 3-kinase and AKT2, and induces apoptosis in OVCAR3 and OVCAR8 cells. The cells were seeded into 60 mm dishes and grown for 24 h and then treated with wortmannin (200 nM) or LY294002 (40 μM) for 12 h. Apoptosis was determined by TdT-mediated dUTP nick end labeling (Tunel) using an *in situ* cell death detection kit (Boehringer). To detect DNA fragmentation, cellular DNA was prepared using the DNA kit (Qiagen). The DNA was analysed on 1.5% agarose gel and visualized by ethidium bromide staining

1997; Liu *et al*., 1998). Active Ras and Src significantly induce AKT2 activation (Liu *et al*., 1998). Recent studies have shown that Akt activity is regulated by *PTEN*, which reduces intracellular levels of PI-3,4,5,-P_3 in cells by converting PI-3,4,5-P_3 to PI-4,5-P_2 and, thus, inhibits PI 3-kinase/Akt signaling pathway (Stambolic *et al*, 1998; Aman *et al*., 1998). Therefore, AKT2 activation in human ovarian cancer may result from; (a) PI 3-kinase activation; (b) *Pten* mutation; (c) overexpression of AKT2; (d) alterations of growth factor receptor, such as overexpression or mutation of EGFR; (e) *ras* mutation; or (f) active mutation of the *AKT2* gene. In this report, we document that nearly half of cases with AKT2 activation (15/33) display PI 3-kinase activation, which supports a recent observation of PI 3-kinase activation in ovarian cancer cell lines (Shayesteh *et al*., 1999). Down regulation of PTEN was detected in three non-endometrioid cases of ovarian tumors. Interestingly, we did not observe either lack of PTEN expression or AKT2 activation in all 10 endometrioid adenocarcinomas examined, implying that the *Pten* may not be mutated in these specimens. We have also performed single strain conformation polymorphism analyses of the tumors carrying activated AKT2. No *AKT2* mutation was observed. The cases exhibiting AKT2 activation express variable levels of AKT2 protein, suggesting that expression of AKT2 protein is required for activation of AKT2 in ovarian tumor.

The fact that three members of Akt family are down stream targets of PI 3-kinase and are regulated by similar mechanism suggests that AKT1 and AKT3 may also be activated in human ovarian cancer. In this study, we focused on activation of AKT2 in primary ovarian cancer. However, we have shown activation of AKT1 and PI 3-kinase but not AKT2 in five ovarian cancer specimens, which express high levels of AKT1 but very low levels of AKT2 (Figure 3). Additional experiments to evaluate the significance of this finding are in progress.

We have previously demonstrated that an activated Ras significantly activates AKT2. We have also recently documented that farnesyltransferase inhibitor (FTI)-277, an anticancer drug blocking the posttranslational farnesylation of oncogenic Ras, inhibits PI 3-kinase and AKT2 activity and induces apoptosis in

AKT2 overexpressing cell lines (Jiang *et al*., 2000). Overexpression of AKT2, but not Ras, sensitizes NIH3T3 cells to FTI-277. However, FTI-277 is not a direct inhibitor of PI 3-kinase and AKT2. In this study, we demonstrate that direct inhibition of PI 3-kinase/Akt pathway by wortmannin or LY294002 induces apoptosis in ovarian cancer cell lines with PI 3-kinase/AKT2 activation. Moreover, we have also shown that the majority of tumors with activated AKT2 are high grade and late stage. These data indicate that activation of PI 3-kinase/Akt may play a pivotal role in development of human ovarian cancer, especially in tumor progression, and that PI 3-kinase/Akt pathway could be an important target for intervention of this malignancy. Future studies are required to define the role of Akt activation in ovarian malignant transformation.

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Phosphatidylinositol 3-Kinase/Akt Pathway Regulates Tuberous Sclerosis Tumor Suppressor Complex by Phosphorylation of Tuberin*

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Normal cellular functions of hamartin and tuberin, encoded by the *TSC1* and *TSC2* tumor suppressor genes, are closely related to their direct interactions. However, the regulation of the hamartin-tuberin complex in the context of the physiologic role as tumor suppressor genes has not been documented. Here we show that insulin or insulin growth factor (IGF) 1 stimulates phosphorylation of tuberin, which is inhibited by the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 but not by the mitogen-activated protein kinase inhibitor PD98059. Expression of constitutively active PI3K or active Akt, including Akt1 and Akt2, induces tuberin phosphorylation. We further demonstrate that Akt/PKB associates with hamartin-tuberin complexes, promoting phosphorylation of tuberin and increased degradation of hamartin-tuberin complexes. The ability to form complexes, however, is not blocked. Akt also inhibits tuberin-mediated degradation of $p27^{kip1}$, thereby promoting CDK2 activity and cellular proliferation. Our results indicate that tuberin is a direct physiological substrate of Akt and that phosphorylation of tuberin by PI3K/Akt is a major mechanism controlling hamartin-tuberin function.

Tuberous sclerosis complex (TSC)[1] is an autosomal dominant disorder and is characterized by the presence of hamartomas in many organs such as brain, skin, heart, lung, and kidney (1). It is caused by mutation of either the *TSC1* or *TSC2* tumor suppressor gene (2–5). *TSC1* encodes a protein, hamartin, containing two coiled-coil domains that have been shown to mediate binding to hamartin (6). The *TSC2* gene codes for tuberin, which contains a small region of homology to the rap1GTPase-activating protein, rap1GAP (7). These two proteins function within the same pathway(s) regulating cell cycle, cell growth, adhesion, and vesicular trafficking (4, 5). However, the regulation of hamartin and tuberin in the context of physiologic role as tumor suppressor genes has not been documented.

Among the various properties of these two proteins, the ability to interact and to form stable complex has been the most consistent finding. This led to the hypothesis that hamartin and tuberin function as a complex and that factors regulating their interaction are important in understanding physiologic roles. There is evidence to suggest that phosphorylation of tuberin may be a major mechanism of regulation of the hamartin-tuberin complex (8, 9). However, the kinases that are responsible for phosphorylation of this complex are currently unknown. Recent *Drosophila* genetic studies showed that *dTsc1* and *dTsc2* play an important role in the insulin/dPI3K/dakt signal transduction pathway by demonstrating that reduced cell size and cell proliferation caused by either mutations in *dINR* and *dakt* or by overexpression of *dPTEN* are overridden by homozygous mutants of *dTsc1* or *dTsc2*. This implies that *dTsc1* and *dTsc2* are either direct downstream targets of dakt or on a parallel pathway of the insulin cascade downstream from dakt (10–13). Akt, also known as protein kinase B (PKB), represents a subfamily of the serine/threonine protein kinase. Three isoforms of Akt have been identified including Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, all of which are activated by growth factors and insulin in a PI3K-dependent manner and are inhibited by PTEN tumor suppressor (14). Akt regulates a wide spectrum of cell functions, including cell survival, cell growth, differentiation, angiogenesis, and glucose metabolism, through phosphorylation of a number of proteins that contain the R*X*R*XX*S/T motif (14–16).

Here we show that Akt physically interacts with and phosphorylates tuberin, leading to degradation of the hamartin-tuberin complex and $p27^{kip1}$ without interfering with hamartin-tuberin complex formation. Moreover, IGF1 and insulin induce tuberin phosphorylation, which is mediated by the PI3K/Akt pathway but not by the MAPK pathway. As a result, cyclin-dependent kinase (CDK) 2 activity, DNA synthesis, and S phase of the cell cycle are elevated. We thus have identified Akt as a major tuberin kinase to negatively regulate hamartin-tuberin tumor suppressor function by inducing degradation.

EXPERIMENTAL PROCEDURES

Plasmids—The cytomegalovirus-based expression constructs encoding wild type, constitutively active, and dominant negative Akt, Myc-TSC1, and TSC2-Xpress have been described (3, 17). TSC2-7A and

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[1] The abbreviations used are: TSC, tuberous sclerosis complex; PI3K, phosphatidylinositol 3-kinase; HA, hemagglutinin; IGF, insulin-like growth factor; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; HEK, human embryonic kidney; GST, glutathione *S*-transferase; CDK, cyclin-dependent kinase.

TSC2-7D mutant constructs were created using a QuikChange multiple site-directed mutagenesis kit (Stratagene). Constitutively active PI3K (p110*) was provided by Julian Downward (London, UK).

Cell Culture, Transfection, and Flow Cytometry—Human embryonic kidney (HEK) 293 and HeLa cells were obtained from the American Type Culture Collection. EEF4 (TSC2-positive) and EEF8 (TSC2-negative) cells were derived from Eker rat embryos homozygous for the wild type and the Eker-mutant TSC2 gene, respectively (8). All cells were grown either in Dulbecco's modified Eagle's medium or in RPMI 1640 medium, both supplemented with 10% calf serum and antibiotics. Cell transfections were performed using LipofectAMINE Plus. For cytofluorometric analyses, cells were harvested by trypsinization, fixed, and analyzed on a FACScan.

Immunoprecipitation, Immunoblotting, and in Vitro Kinase Assay—For immunoprecipitation, lysates were incubated with the appropriate antibody (as noted in the figure legends) in the presence of protein A-protein G (2:1)-agarose beads. The beads were washed with lysis buffer. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Detection of antigen-bound antibody was carried out with the ECL System. Protein kinase assays were performed as described previously (18).

Pulse-chase Experiments—Prior to radioactive labeling normal culture medium was removed, and cells were washed twice with phosphate-buffered saline and refed with minimum Eagle's medium lacking methionine but supplemented with 10% dialyzed fetal bovine serum and 300 μCi of Tran^{35}S-label per plate. After 60 min of labeling, cells were lysed and immunoprecipitated with anti-TSC1, -TSC2, or -p27 antibody. The immunoprecipitates were separated by SDS-PAGE gel. Gels were dried and autoradiographed. Quantification of bands was performed with a PhosphorImager.

In Vivo [^{32}P]orthophosphate Cell Labeling—COS7 cells were transfected with pcDNA3-TSC2 together with or without constitutively active Akt and labeled with [^{32}P]orthophosphate (0.5 mCi/ml) in minimum Eagle's medium without phosphate for 4 h. Tuberin was immunoprecipitated with anti-TSC2 antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated tuberin was detected by autoradiography.

RESULTS

Tuberin Is a Physiological Substrate of Akt—Recent studies demonstrated that tuberin is phosphorylated at serine and tyrosine residues in response to serum, phosphatase inhibitors, and anisomycin and that the phosphorylated tuberin regulates its interaction with hamartin (8, 9). However, the kinases that are responsible for phosphorylation of tuberin are currently unknown. Because tuberin contains seven Akt phosphorylation consensus sites that are very conserved between human, rat, and mouse as well as four that are also found in *Drosophila* (Fig. 1*a*), we examined the possibility of Akt phosphorylation of tuberin. *In vivo* [^{32}P]orthophosphate cell-labeling experiments revealed that constitutively active Akt and IGF1-induced Akt significantly phosphorylate tuberin (Fig. 1*b*). To explore which sites on tuberin are potentially phosphorylated by Akt, *in vitro* kinase assay was carried out using wild type and mutant (converting S/T to alanine) GST fusion proteins for each of seven Akt putative phosphorylation sites as substrate. As shown in Fig. 1c, Akt can highly phosphorylate fusion proteins containing all seven serine and threonine sites of tuberin but not their mutants. We therefore conclude that tuberin is a physiological substrate of Akt.

The PI3K/Akt Pathway, but Not the MAPK Pathway, Mediates Insulin, IGF1, and Serum-induced Tuberin Phosphorylation—Because genetic studies of the *dTsc complex* in *Drosophila* have demonstrated that *dTsc1/dTsc2* antagonize insulin signaling in cell growth (10–13), we next examined whether insulin and IGF1 induce hamartin-tuberin phosphorylation and whether Akt mediates this action. Western blotting analyses showed that tuberin, but not hamartin, was phosphorylated upon insulin, IGF1, or serum stimulation in HeLa cells as demonstrated by gel mobility shift (Fig. 1*d*). The phosphorylation was abrogated by treatment with phosphatase PP2A or PI3K inhibitors, LY294002, and wortmannin, but not by MAPK

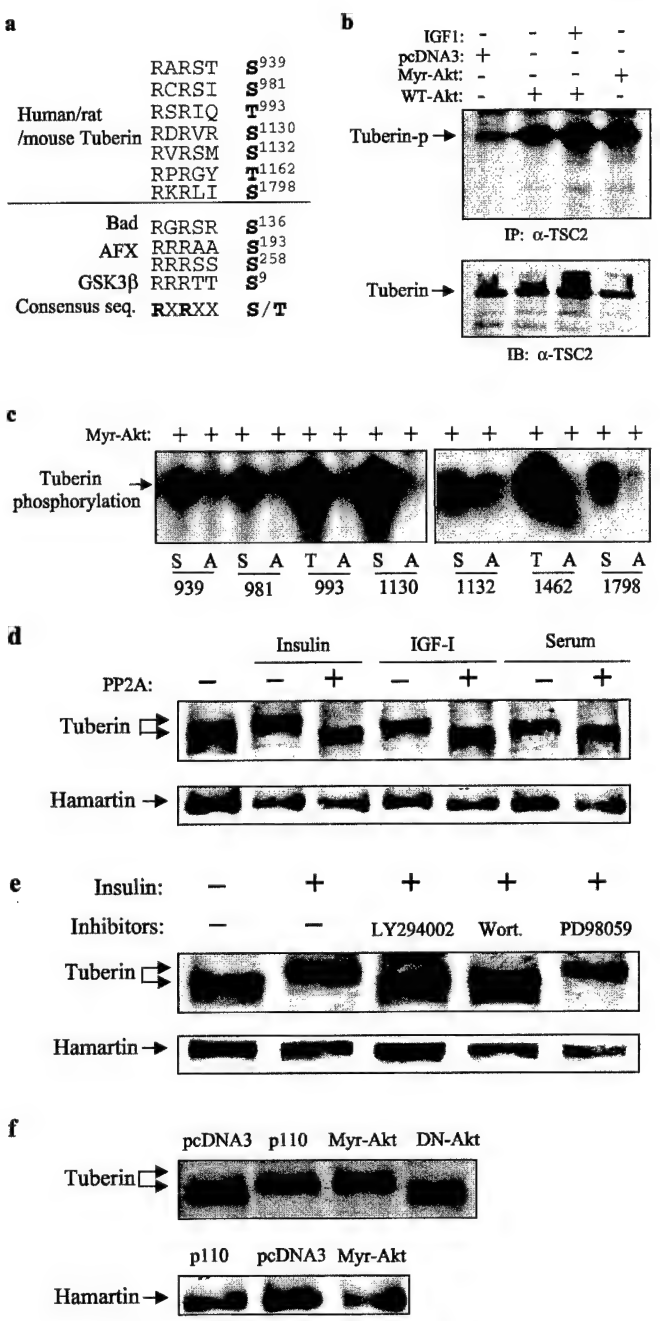

FIG. 1. **Akt phosphorylates tuberin *in vitro* and *in vivo* and mediates insulin- and IGF1-induced tuberin phosphorylation.** *a*, comparison of the putative Akt phosphorylation sites in tuberin with the sequences of phosphorylation sites of known Akt substrates. The phosphorylated residues are labeled by number, and a consensus sequence is denoted below. *b*, *in vivo* [^{32}P]orthophosphate labeling HeLa cells transfected with constructs immunoprecipitated with anti-TSC2 antibody are indicated at the *top*. Immunoprecipitates were separated by SDS-PAGE, transferred to membrane, exposed to the film (*upper panel*), and detected by anti-TSC2 antibody (*bottom panel*). *WT-Akt*, wild type Akt. *c*, *in vitro* kinase assay analysis of constitutively active Akt (*Myr-Akt*) immunoprecipitates using each of the GST-fused seven Akt phosphorylation sites and their alanine (*A*) mutants as substrate indicated at the bottom. *d–f*, Western blot analysis of tuberin in HeLa cells that were serum-starved overnight and stimulated with or without insulin, IGF1, or serum for 15 min. Tuberin was immunoprecipitated, treated with phosphatase PP2A, and immunoblotted with anti-tuberin antibody. Electrophoretic mobility shift of tuberin, *i.e.* the phosphorylated form of tuberin, was observed upon insulin, IGF1, or serum stimulation and was abrogated by treatment with PP2A (*d*) or PI3K inhibitors (*LY294002* and *wortmannin*) for 20 min (*e*). *f*, HeLa cells were transfected with constitutively active p110, Akt, and DN-Akt and immunoblotted with anti-TSC2 (*top*) and -TSC1 (*bottom*) antibodies.

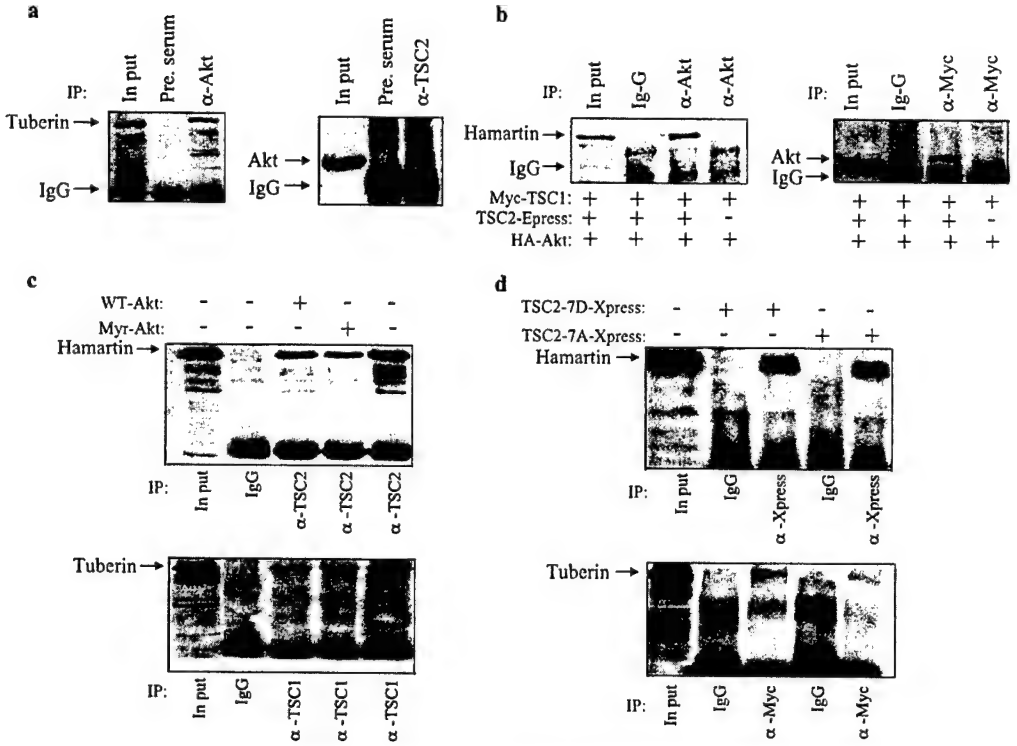

FIG. 2. **Akt interacts with tuberin but does not interfere with hamartin-tuberin complex formation.** *a*, Western blot analyses of immunoprecipitates prepared from HeLa cells with anti-Akt antibody and detected with anti-TSC2 antibody (*left*) or *vice versa* (*right*). Preimmune serum (*Pre. serum*) was used as negative control, and total cell lysate (*In put*) indicates expression of TSC2 and Akt. *b*, Akt is indirectly associated with hamartin. HEK293 cells were transfected with plasmids as indicated at the *bottom*. Immunoprecipitations were prepared with polyclonal Akt antibody and detected with monoclonal anti-Myc antibody (*left*) or *vice versa* (*right*). *c*, HeLa cells were transfected with wild type, constitutively active Akt, or pcDNA3 vector alone. Immunoprecipitations were carried out with antibodies indicated at the *bottom* of each panel and detected with anti-TSC1 (*top*) or -TSC2 (*bottom*) antibody. *d*, HEK293 cells were transfected with TSC2-7D-Xpress or TSC2-7A-Xpress, immunoprecipitated with anti-Xpress antibody, and detected with anti-Myc antibody (*top*) or *vice versa* (*bottom*).

inhibitor PD98059 (Fig. 1*e*). Furthermore, expression of constitutively active PI3K (p110*) and active Akt significantly induced the phosphorylation of tuberin (Fig. 1*f*). These data indicate that the function of TSC tumor suppressors is regulated by mitogenic growth factor IGF1 and insulin through the PI3K/Akt, but not the MAPK, pathway.

Akt Interacts with Tuberin and Hamartin—To assess whether Akt physically associates with the hamartin-tuberin complex, coimmunoprecipitation experiments were performed in HeLa cells. As seen in Fig. 2*a*, Akt directly bound to tuberin. Because hamartin and tuberin function as a complex and Akt associates with tuberin, we assumed that Akt could indirectly interact with hamartin. To test this hypothesis, HEK293 cells were transfected with Myc-TSC1, HA-Akt, and/or TSC2-Xpress. Coimmunoprecipitation experiments revealed that interaction between Akt and hamartin was only detected in the cells transfected with Myc-TSC1/TSC2-Xpress/HA-Akt but not with Myc-TSC1/Akt (Fig. 2*b*), indicating that Akt binding to hamartin is mediated by tuberin. Because tuberin binds to Akt and is phosphorylated by Akt, we conclude that tuberin is a direct downstream target of Akt.

The Hamartin-Tuberin Complex Is Not Disrupted by Akt Phosphorylation of Tuberin—Because previous studies have suggested that phosphorylation of tuberin regulates its interaction with hamartin (8, 9), we next examined whether Akt interferes with hamartin-tuberin complex formation. Coimmunoprecipitation revealed that expression of wild type and constitutively active Akt in HeLa cells did not disrupt the interaction between hamartin and tuberin (Fig. 2*c*), despite the fact that hamartin and tuberin function as a complex. Moreover, phosphomimic TSC2-7D-Xpress and nonphosphorylatable TSC2-7A-Xpress, prepared by converting seven Akt phosphorylation sites of tuberin into aspartic acid and alanine, respectively, were transfected into HeLa cells. Immunoblotting analyses of TSC2-7D-Xpress and TSC2-7A-Xpress immunoprecipitates showed that both mutant forms of tuberin still bound to hamartin (Fig. 2*d*), indicating that Akt phosphorylation of tuberin did not hamper the interaction between hamartin and tuberin.

Akt Phosphorylation of Tuberin Induces Degradation of Hamartin and Tuberin—Strikingly, we observed that expression of constitutively active Akt significantly down-regulated hamartin and tuberin in a dose-dependent manner, *i.e.* protein levels of hamartin and tuberin progressively declined when the cells were transfected with increasing amounts of constitutively active Akt. Accordingly, the protein amount of hamartin and tuberin in the complex was also decreased (Fig. 3*a*). To exclude the possibility of Akt down-regulation of hamartin and tuberin resulting from inhibition of the *TSC1* and *TSC2* gene transcription, Northern blot analyses were performed and showed that mRNA levels of *TSC1* and *TSC2* did not change in HeLa cells transfected with constitutively active Akt as compared with the cells transfected with pcDNA3 vector alone (Fig. 3*e*). Because Akt has been shown to activate rather than inhibit translation initiation through regulation of FRAP/mTOR/4E-BP (14, 15), it is unlikely that Akt regulates hamartin and tuberin at a translational level. Thus, we assumed Akt down-regulation of hamartin and tuberin occurred through protein degradation. Pulse-chase experiments revealed that expression of constitutively active Akt considerably induced hamartin and tuberin degradation (Fig. 3*b*). Moreover, the proteosome inhibitor MG132 attenuated Akt-induced hamartin and tuberin degradation (Fig. 3*c*). These data suggest that Akt down-regulation of hamartin and tuberin is mediated by a post-transla-

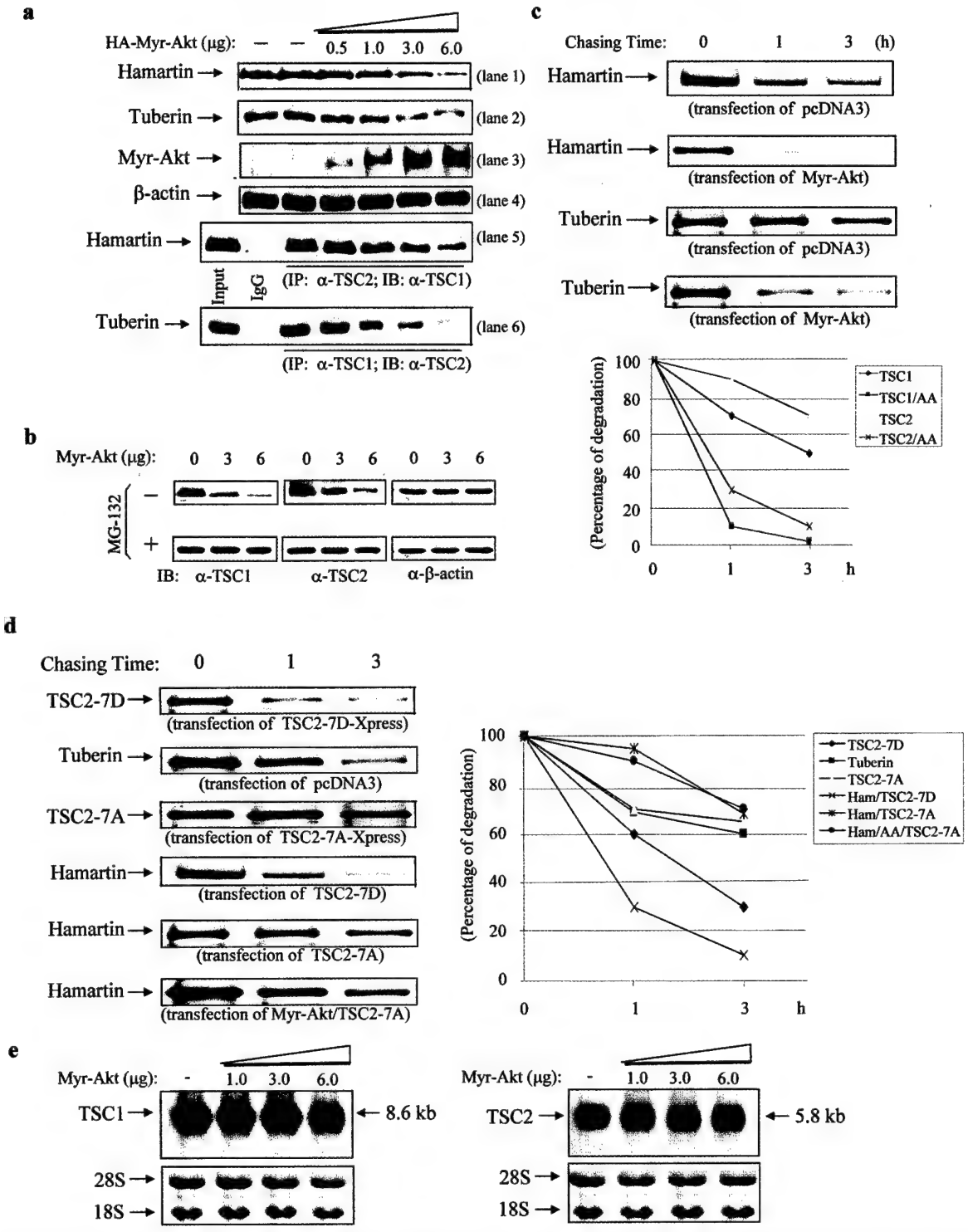

FIG. 3. **Akt phosphorylation of tuberin induces degradation of hamartin and tuberin.** *a*, HeLa cells were transfected with HA-Myr-Akt. A portion of the cell lysate was subjected to Western blot analysis using anti-TSC1 (*panel 1*), -TSC2 (*panel 2*), -HA (*panel 3*), and -β-actin (*panel 4*) antibodies. The rest were immunoprecipitated with anti-TSC2 antibody and detected with anti-TSC1 antibody (*panel 5*) or *vice versa* (*panel 6*). *b*, constitutively active Akt-transfected HeLa cells were pretreated with or without MG132 for 2 h, lysed, and subjected to immunoblotting analyses with anti-TSC1, -TSC2, and -β-actin antibodies. *c*, pulse-chase analyses of degradation of hamartin and tuberin. TSC2-positive EEF4 cells were transfected with plasmids indicated at the *bottom* of each panel, labeled with [^{35}S]methionine, chased at indicated times, and immunoprecipitated with anti-TSC1 or -TSC2 antibodies. The immunoprecipitates were separated by SDS-PAGE, exposed, and quantified. *d*, phosphomimic TSC2-7D promotes and nonphosphorylatable TSC2-7A inhibits the degradation of hamartin and tuberin. TSC2-deficient EEF8 cells were transfected with indicated expression plasmids (*bottom of each panel*) and chased at indicated times after labeling with [^{35}S]methionine. Immunoprecipitations were performed with anti-TSC1, -TSC2, or -Xpress antibodies. Graphical presentations show the normalized density of hamartin and tuberin degradation from 100%. *e*, ectopic expression of constitutively active Akt does not affect mRNA levels of TSC1 and TSC2. HeLa cells were transfected with increasing amounts of constitutively active Akt. After 48 h of transfection, total RNAs were isolated and subjected to Northern blot analyses with [^{32}P]dCTP-labeled TSC1 (*left*) or TSC2 (*right*) cDNA probe. *Bottom panels* indicate equal loadings.

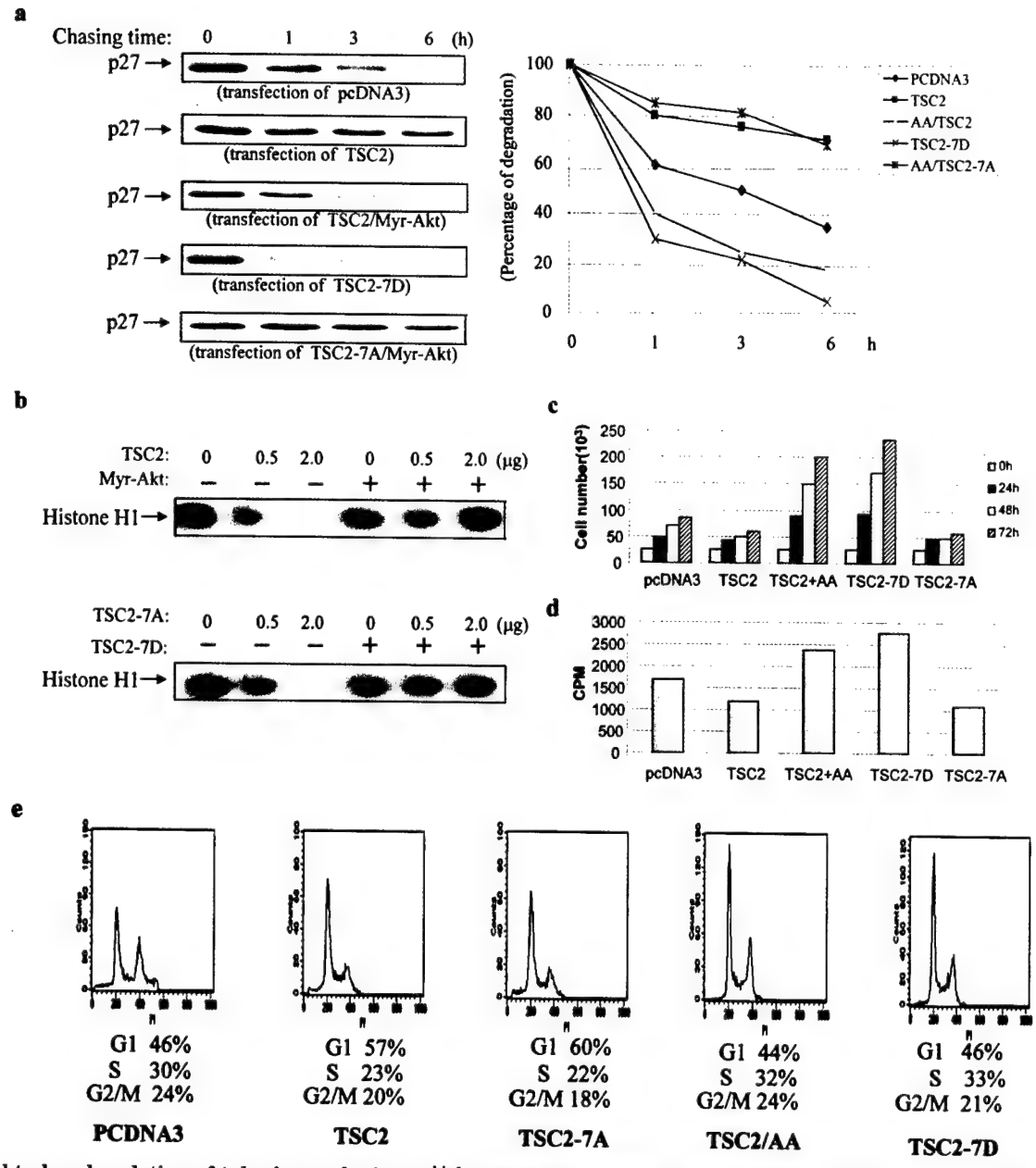

FIG. 4. **Akt phosphorylation of tuberin results in $p27^{kip1}$ degradation and cell proliferation.** *a*, similar to Fig. 4*d*, HeLa cells were transfected with expression constructs indicated at the *bottom* of each panel, labeled with [^{35}S]methionine, chased at indicated times, immunoprecipitated with anti-$p27^{kip1}$ antibody, and separated by SDS-PAGE. Graphical presentations show the normalized density of $p27^{kip1}$ degrading from 100%. *b*, *in vitro* CDK2 kinase assay analyses of cyclin E-CDK2 immunoprecipitates prepared anti-cyclin E antibody from HeLa cells transfected with the indicated plasmids. *c*, cell growth. EEF8 TSC2-deficient cells were transfected with the indicated expression constructs (*bottom of diagram*) in triplicate 6-well plates. Cells were counted using a Coulter cell counter at different times. *d*, thymidine incorporation. EEF8 TSC2-deficient cells were transfected with the indicated plasmid (*bottom of diagram*) and labeled with [^{3}H]thymidine for 12 h. Data were obtained from four independent experiments. *e*, flow cytometry. HeLa cells were co-transfected with CD20 and the expression constructs indicated at the *bottom*. After 48 h, cells were harvested, stained with anti-CD20-FITC antibody and propidium iodide, and analyzed by a fluorescence-activated cell sorter.

tional modification mechanism in which the proteosome pathway is involved.

To examine whether Akt-induced hamartin-tuberin degradation depends upon Akt phosphorylation of tuberin, EEF8 TSC2-deficient cells were transfected with wild type TSC2, phosphomimic TSC2-7D, or nonphosphorylatable TSC2-7A. Western blotting and pulse-chase analyses revealed that TSC2-7D was degraded more rapidly than wild type TSC2, whereas TSC2-7A became more stable. Expression of TSC2-7D promoted hamartin degradation, whereas TSC2-7A stabilized hamartin and inhibited Akt-induced hamartin degradation (Fig. 3*d*), indicating that Akt phosphorylation of tuberin is required for degradation of the hamartin-tuberin complex. Previous studies have shown that tuberin functions as a cytosolic chaperone protein to prevent hamartin self-aggregation and maintain the tuberin-hamartin complex in a soluble form (9, 19). However, we did not observe that Akt phosphorylation of tuberin affected its chaperone function (data not shown).

Akt Phosphorylation of Tuberin Leads to Down-regulation of $p27^{kip1}$ and Cell Proliferation—The results from studying altered expression of either *TSC1* or *TSC2* have demonstrated that both hamartin and tuberin inhibit cell growth and cell size in mammalian (4, 5) and *Drosophila* (10–12), respectively. The mechanism that has been characterized so far is that overexpression of hamartin and tuberin induces the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ through inhibition of

its degradation (20, 21). To examine the effects of Akt phosphorylation of tuberin on $p27^{kip1}$ expression, pulse-chase analyses were performed with TSC2-deficient EEF8 cells that were transfected with TSC2, TSC2/Mry-Akt, TSC2-7D, or TSC2-7A. As shown in Fig. 4*a*, expression of constitutively active Akt abrogated the ability of stabilization of $p27^{kip1}$ by tuberin. $P27^{kip1}$ degraded rapidly in phosphomimic TSC2-7D-transfected cells as compared with the cells expressing wild type TSC2/constitutively active Akt. In contrast, the cells expressing TSC2-7A exhibited similar degradation rate of $p27^{kip1}$ to wild type TSC2-transfected cells. Moreover, expression of TSC2-7A abrogated constitutively active Akt-induced $p27^{kip1}$ degradation (Fig. 4*a*). These data indicate that degradation of $p27^{kip1}$ is regulated by Akt phosphorylation of tuberin.

Because G_1/S CDK 2 is a major target of $p27^{kip1}$ (21), we next examined whether Akt overrides tuberin-inhibited CDK2 activity. Consistent with previous reports (21, 22), expression of wild type *TSC2* inhibited CDK2 activity in a dose-dependent manner. However, constitutively active Akt abrogated *TSC2*-inhibited CDK2 activity (Fig. 4*b*). Phosphomimic TSC2-7D lost the ability to inhibit CDK2 activity, whereas expression of nonphosphorylatable TSC2-7A displayed the same effects as wild type TSC2 (Fig. 4*b*).

Because CDK2 is a major regulator of cell growth and G_1/S transition of the cell cycle, we further examined the effects of Akt phosphorylation of tuberin on cell proliferation measured by cell growth and thymidine incorporation. As shown in Fig. 4, *c* and *d*, expression of TSC2 or TSC2-7A in TSC2-deficient EEF8 cells inhibited cell growth and DNA synthesis as compared with the cells transfected with vector alone. However, cells expressing constitutively active Akt or phosphomimic TSC2-7D significantly enhanced cell growth and thymidine incorporation. Consistent with previous findings, the number of cells at the G_0-G_1 phase of the cell cycle was increased in the cells expressing TSC2. Expression of TSC2-7A displayed a similar effect on cell cycle. In contrast, constitutively active Akt overrode wild type TSC2 action. The cell number of the S phase was increased in constitutively active Akt/TSC2- or phosphomimic TSC2-7D-transfected cells (Fig. 4*e*). These data indicate that Akt phosphorylated tuberin lost its tumor suppressor function at least in part by inducing $p27^{kip1}$ degradation.

DISCUSSION

Recent studies have demonstrated that phosphorylation of hamartin and/or tuberin may play an important role in the formation of the tuberin-hamartin complex. Tuberin is phosphorylated at serine and tyrosine residues, and a disease-related TSC2 tyrosine 1571 mutation (Y1571H) nearly abolishes tuberin tyrosine phosphorylation and disrupts tuberin-hamartin binding, implying that the phosphorylation of tyrosine 1571 of TSC2 is required for tuberin-hamartin complex formation (8, 9). Our study, however, shows that phosphorylation of tuberin by Akt and mitogenic factors (insulin and IGF1) abrogates hamartin-tuberin tumor suppressor activity without interfering with binding but by inducing degradation of both proteins through the proteosome pathway. Therefore, we provide a new paradigm for regulation of the TSC1/TSC2 tumor suppressor pathway.

In addition to the Forkhead transcription factor family (16, 22), tuberin is the second Akt downstream target that has been uncovered by genetic studies so far. In this study, we present molecular evidence that tuberin is a direct physiological substrate of Akt by demonstrating that Akt binds to and phosphorylates tuberin. It has been documented that Akt induces cell cycle progression and cell proliferation through transcription repression and degradation of $p27^{kip1}$ (23, 24). Akt inhibition of $p27^{kip1}$ transcription is achieved by Akt phosphorylation of a Forkhead transcription factor, AFX, leading to the decrease of $p27^{kip1}$ promoter activity (24). However, the mechanism of Akt degradation of $p27^{kip1}$ is unclear. Tuberin was revealed to stabilize $p27^{kip1}$ by maintaining $p27^{kip1}$ in the nucleus (20). We observed in this study that Akt attenuates the tuberin action but does not induce translocation of $p27^{kip1}$ from nuclear to cytoplasm (data not shown). Previous studies have shown that three isoforms of Akt share almost the same upstream regulators and downstream targets. Similarly, we have observed that Akt1, Akt2, and Akt3 all phosphorylate and interact with tuberin, even though Akt2 displays a slightly higher binding affinity to tuberin. The model in Fig. 5 illustrates the mechanism through which the PI3K/Akt pathway mediates insulin and IGF1 signals to down-regulate hamartin-tuberin function by phosphorylation of tuberin. Our results define a possible new mechanism through which Akt induces cell proliferation and transformation by inhibiting TSC1/TSC2 tumor suppressor functions.

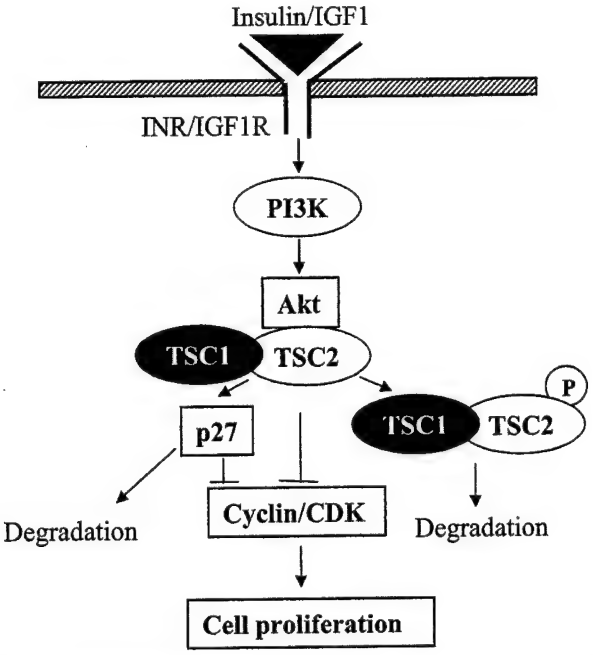

FIG. 5. **Schematic illustration of negative regulation of the TSC tumor suppressor complex by PI3K/Akt.**

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Inhibition of JNK by Cellular Stress- and Tumor Necrosis Factor α-induced AKT2 through Activation of the NFκB Pathway in Human Epithelial Cells*

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Previous studies have demonstrated that AKT1 and AKT3 are activated by heat shock and oxidative stress via both phosphatidylinositol 3-kinase-dependent and -independent pathways. However, the activation and role of AKT2 in the stress response have not been fully elucidated. In this study, we show that AKT2 in epithelial cells is activated by UV-C irradiation, heat shock, and hyperosmolarity as well as by tumor necrosis factor α (TNFα) through a phosphatidylinositol 3-kinase-dependent pathway. The activation of AKT2 inhibits UV- and TNFα-induced c-Jun N-terminal kinase (JNK) and p38 activities that have been shown to be required for stress- and TNFα-induced programmed cell death. Moreover, AKT2 interacts with and phosphorylates IκB kinase α. The phosphorylation of IκB kinase α and activation of NFκB mediates AKT2 inhibition of JNK but not p38. Furthermore, phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 significantly enhances UV- and TNFα-induced apoptosis, whereas expression of constitutively active AKT2 inhibits programmed cell death in response to UV and TNFα stimulation with an accompanying decreased JNK and p38 activity. These results indicate that activated AKT2 protects epithelial cells from stress- and TNFα-induced apoptosis by inhibition of stress kinases and provide the first evidence that AKT inhibits stress kinase JNK through activation of the NFκB pathway.

Exposure of cells to environmental stress results in the activation of several signal transduction pathways including the MEKK4/MKK7/JNK,[1] MKK3/MKK6/p38, and IκB kinase (IKK)/IκB/NFκB cascades. Stress-induced clustering and internalization of cell surface receptors, such as those for platelet-derived growth factor, tumor necrosis factor α (TNFα), epidermal growth factor, and insulin-like growth factor 1 (IGF1), mediate stress-kinase activation (1–3). Recent studies suggest that nearly all stress stimuli activate phosphatidylinositol 3-kinase (PI3K) (1), and of the downstream targets of PI3K, AKT is thought to play an essential role in the cellular response to stress.

AKT, also termed protein kinase B or RAC kinase, represents a family of PI3K-regulated serine/threonine kinases (4, 5). Three different isoforms of AKT have been identified, AKT1/protein kinase Bα (AKT1), AKT2/protein kinase Bβ (AKT2), and AKT3/protein kinase Bγ (AKT3), all of which are activated by growth factors in a PI3K-dependent manner (4–9). Full activation of the AKTs requires their phosphorylation at Thr^{308} (AKT1), Thr^{309} (AKT2), or Thr^{305} (AKT3) in the activation loop and Ser^{473} (AKT1), Ser^{474} (AKT2), or Ser^{472} (AKT3) in the C-terminal activation domain (9). AKT1, the most studied isoform, which was originally designated as AKT, suppresses apoptosis induced by a variety of stimuli, including growth factor withdrawal and loss of cell adhesion. Possible mechanisms by which AKT1 promotes cell survival include phosphorylation and inactivation of the proapoptotic proteins BAD and caspase-9 (10, 11). AKT1 also phosphorylates and inactivates the Forkhead transcription factors, an event that results in the reduced expression of the cell cycle inhibitor, $p27^{Kip1}$, and the Fas ligand (12–14). Via phosphorylation of IKK, AKT1 also activates NFκB, a transcription factor that has been implicated in cell survival (15, 16).

Two separate studies demonstrated that AKT1 is activated when NIH 3T3 fibroblasts are stressed in a variety of ways (17, 18). Based on data showing that PI3K inhibitors do not prevent AKT1 activation by stress, these studies concluded that stress-induced AKT1 activation was PI3K-independent. Other studies, however, found that PI3K activity was required for AKT1 activation by heat shock or oxidative stress in Swiss 3T3 cells (19, 20). It has been suggested that certain cellular stresses activate AKT1 and AKT3 but not AKT2 (19), a finding that is consistent with the different functions of the AKTs as revealed by studies of mice lacking AKT1 or AKT2 (21–23). Nevertheless, activation of AKT2 by stress and the role of AKT2 in the stress response have yet to be fully explored. The data presented here show that AKT2 is significantly activated by stress stimuli (*e.g.* UV irradiation, heat shock, and hyperosmolarity) and by TNFα in human epithelial cells but not in fibroblasts. Stress-induced AKT2 activation in epithelial cells is completely blocked by inhibitors of PI3K. When activated by stress, AKT2 inhibits JNK and p38 activities that are required for stress-induced apoptosis. In addition, AKT2 binds to and phosphoryl-

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[1] The abbreviations used are: JNK, c-Jun N-terminal kinase; TNFα, tumor necrosis factor α; IGF1, insulin-like growth factor 1; PI3K, phosphatidylinositol 3-kinase; HA, hemagglutinin; IKK, IκB kinase; NIK, NFκB-inducing kinase; GST, glutathione *S*-transferase; HEK, human embryonic kidney; TUNEL assay, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay.

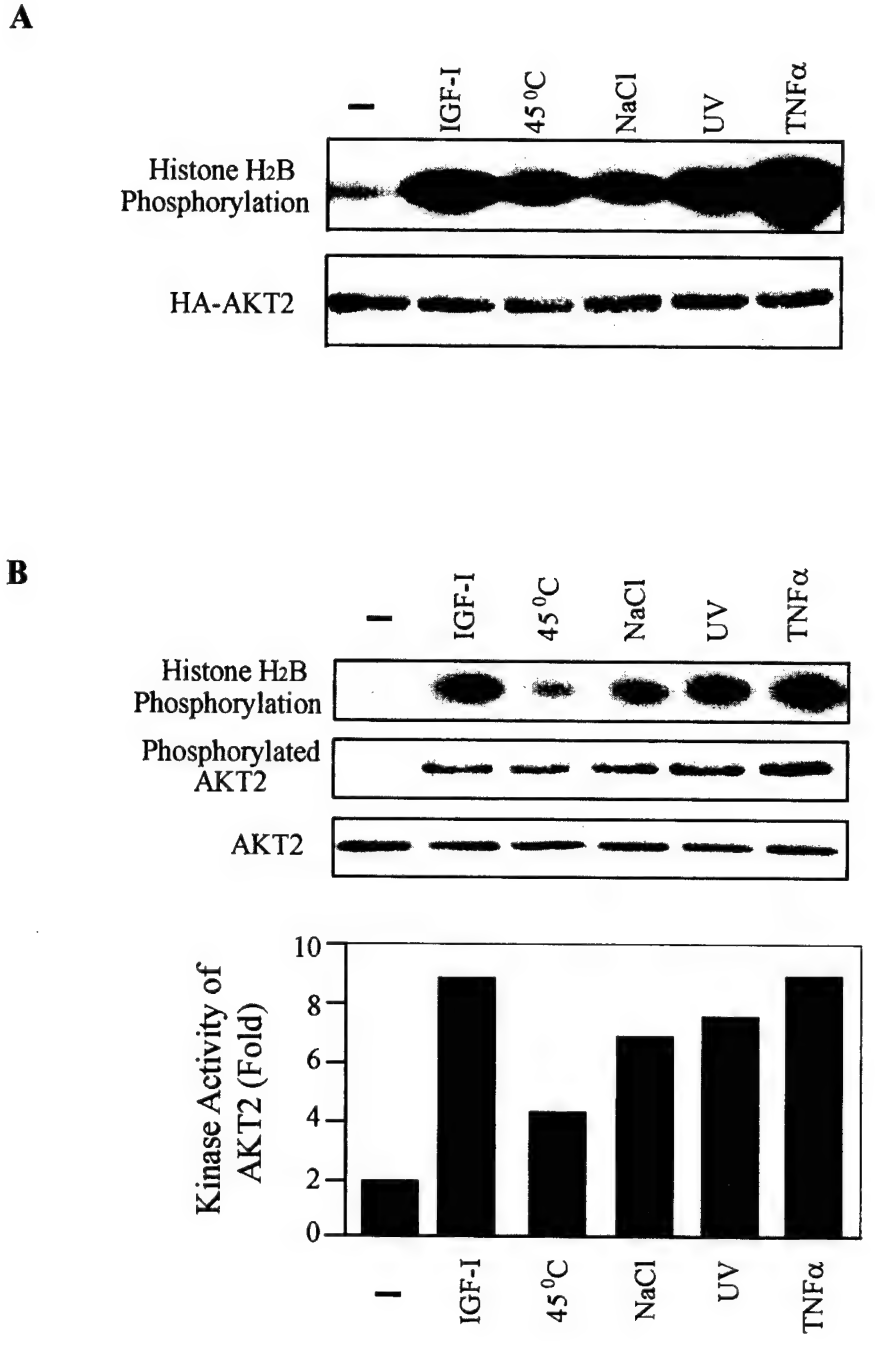


FIG. 1. **AKT2 is activated by cellular stress and TNFα.** *A*, *in vitro* kinase assay of AKT2 immunoprecipitates prepared from A2780 cells transiently transfected with HA-AKT2. Cells were exposed to 100 ng/ml IGF-1 (15 min), heat shock (45 °C for 20 min), 0.4 M NaCl (15 min), 40 J/m^2 UV-C (254 nm), or TNFα 20 ng/ml (15 min), and AKT2 activity was determined by *in vitro* kinase assay using histone H2B as substrate. *B*, OVCAR3 cells were treated with the indicated stimuli and immunoprecipitated with anti-AKT2 antibody. The immunoprecipitates were subjected to *in vitro* kinase assay (*upper*) and Western blotting analyses with anti-phospho-Ser473 AKT (*middle*), or anti-AKT2 (*lower*) antibody. The *bottom panel* shows relative AKT2 kinase activity quantified by phosphorimaging. Each experiment was repeated three times.

ates IKKα and, consequently, activates NFκB, resulting in inhibition of programmed cell death in response to stress stimuli. Moreover, AKT2-induced NFκB activation is required for the inhibition of JNK, but not p38, activity.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfection, and Stimulation—The human epithelial cancer cell lines, A2780, OVCAR3, and human embryonic kidney (HEK) 293 cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cells were seeded in 60-mm Petri dishes at a density of 0.5×10^6 cells/dish. After incubation overnight, the cells were transfected with 2 μg of DNA/dish using LipofectAMINE Plus (Invitrogen). After 36 h of the transfection, the cells were serum-starved overnight and stimulated with UV-C irradiation, heat (45 °C), 0.4 M NaCl, or 20–50 ng/ml TNFα.

Expression Constructs—The cytomegalovirus-based expression constructs encoding wild type HA-AKT2, constitutively active HA-Myr-AKT2, and dominant negative HA-E299K-AKT2 have been described (24). The HA-JNK1 construct was kindly provided by Michael Karin (School of Medicine, University of California at San Diego). GST-c-Jun-(1–79) and pCMV-FLAG-p38 were gifts from Roger J. Davis (School of Medicine, University of Massachusetts). The constructs used in the study of the NFκB pathway were prepared as previously described (25).

Immunoprecipitation and Immunoblotting—Cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM NaPPi, 1 mM sodium vanadate, and 25 mM β-glycerol phosphate. Lysates were centrifuged at 12,000 × *g* for 15 min at 4 °C before immunoprecipitation or Western blotting. Aliquots of the cell lysates were analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1)-agarose beads at 4 °C for 20 min. After the removal of the beads by centrifugation, lysates were incubated with anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals), anti-FLAG antibody (Sigma), or anti-AKT2 antibody (Santa Cruz Biotechnology) in the presence of 30 μl of protein A-protein G (2:1)-agarose beads for 2 h at 4 °C. The beads were washed once with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and 0.5% Triton X-10, twice with phosphate-buffered saline, and once with buffer containing 10 mM Tris-HCl (pH 7.5). 10 mM $MgCl_2$, 10

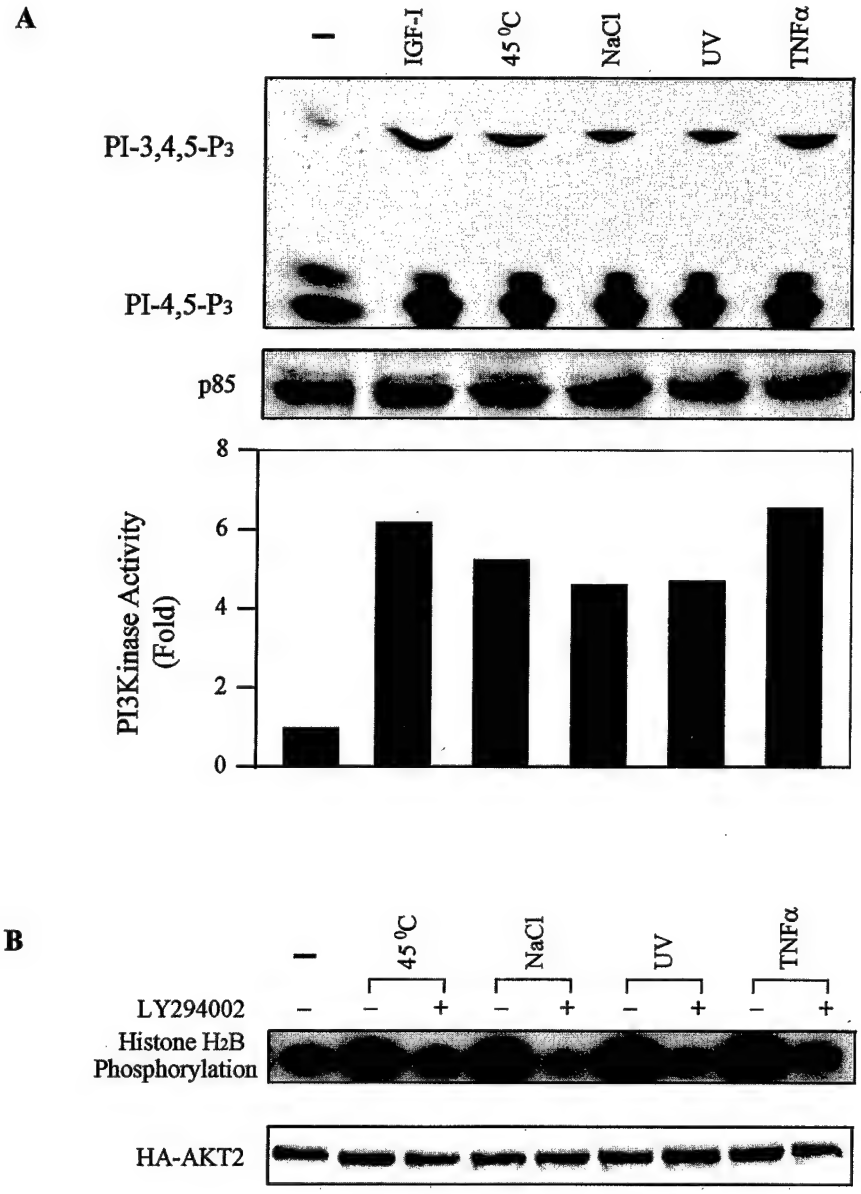


FIG. 2. **Activation of AKT2 by cellular stress and TNFα is PI3K-dependent.** *A*, *in vitro* PI3K assay. HA-AKT2-transfected HEK293 cells were exposed to the indicated stimuli. *Upper panel*, PI3K immunoprecipitates were prepared with anti-pan-p85 antibody and assayed for PI3K activity. The *middle panel* shows the p85 protein level using anti-p85 antibody, and the *bottom panel* represents the relative PI3K activity quantified by phosphorimaging. *B*, HA-AKT2-transfected A2780 cells were treated with LY294002 for 30 min before exposure to indicated stimuli. HA-AKT2 immunoprecipitates were subjected to *in vitro* kinase assay. Results were confirmed by four independent experiments. *PI-3,4,5-P_3*, phosphatidylinositol 3,4,5-trisphosphate; *PI-4,5-P_3*, phosphatidylinositol 4,5-trisphosphate.

mM $MnCl_2$, and 1 mM dithiothreitol, all supplemented with 20 mM β-glycerol phosphate and 0.1 mM sodium vanadate. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates or total cell lysates with the antibodies described above or with the appropriate antibodies as noted in figure legends. Detection of antigen-bound antibody was carried out with the ECL Western blotting Analysis System (Amersham Biosciences).

In Vitro Protein Kinase Assay—Protein kinase assays were performed as previously described (26, 27). Briefly, reactions were carried out in the presence of 10 μCi of [γ-^{32}P] ATP (PerkinElmer Life Sciences) and 3 μM cold ATP in 30 μl of buffer containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. Histone H2B was used as exogenous substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding protein loading buffer, and proteins were separated on SDS-PAGE gels. Each experiment was repeated three times, and the relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics).

PI3K Assay—PI3K was immunoprecipitated from the cell lysates with pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold phosphate-buffered saline, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4), and finally with 10 mM Tris/100 mM NaCl/1 mM EDTA. The presence of PI3K activity in the immunoprecipitates was determined by incubating the beads in reaction buffer (10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP) containing 20 μCi [γ-^{32}P]ATP and 10 μg L-α-phosphatidylinositol 4,5-bisphosphate (Biomol) for 20 min at 25 °C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of $CHCl_3$/MeOH, and phosphorylated products were separated by thin-layer chromatography as previously described (24). The conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate was detected by autoradiography and quantitated with a PhosphorImager.

NFκB Transcriptional Activation Analysis—HEK293 cells were seeded in 60-mm dishes and transfected with 1.5 μg of NFκB reporter plasmid (pElam-luc), 0.8 μg of pSV2-β-gal, and different forms (wild type, constitutively active, or dominant-negative) of HA-AKT2 or vector alone. The total amount of DNA transfected was increased to 6 μg with empty vector DNA. After serum starvation overnight, the cells were treated with UV (40 J/m^2) or TNFα (20 ng/ml) and lysed with 400 μl/dish of reporter lysis buffer (Tropix). The cell lysates were cleared by centrifugation for 2 min at 4 °C. Luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix, respectively). Each experiment was repeated three times.

Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) Assay—AKT2 stably transfected A2780 cells were seeded into 60-mm dishes and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum for 24 h and pretreated with or without LY294002 for 2 h before exposure to UV, heat shock, NaCl, or TNFα. Apoptosis was determined by TUNEL using an *in situ* cell death detection kit (Roche Molecular Biochemicals). The cells were trypsinized, and cytospin preparations were obtained. Cells were fixed with freshly prepared paraformaldehyde (4% in phosphate-

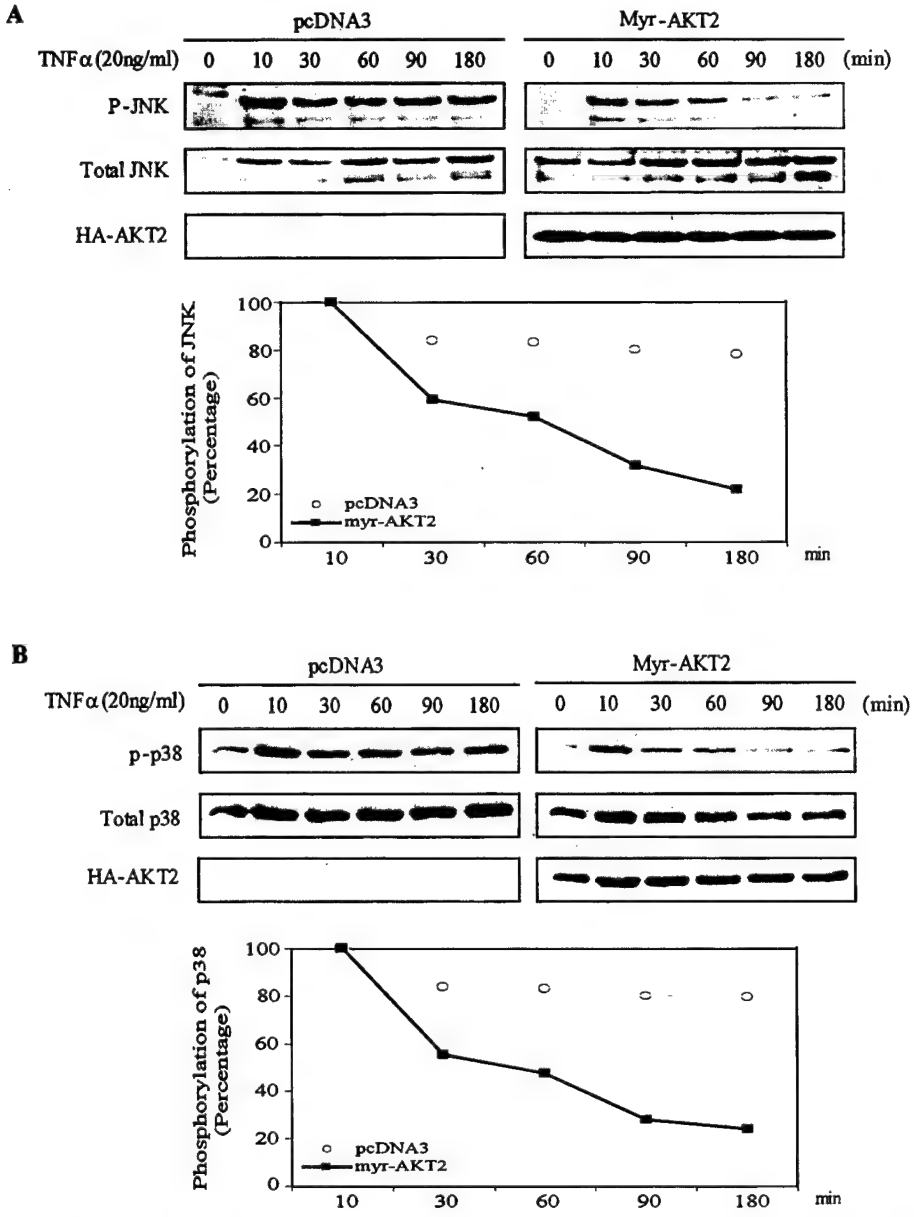


FIG. 3. **AKT2 kinase inhibits UV- and TNFα-induced JNK and p38 activation.** *A*, Western blotting analyses of HEK293 cells transfected with the indicated plasmids. Cells were lysed at indicated times after incubation with TNFα and analyzed with anti-phospho-JNK (*P-JNK, upper*), -total JNK (*middle*), and -HA (*lower*) antibodies. The immunoblotting analyses were repeated three times. *B*, the procedures are the same as *A*, except the membranes were probed with anti-phospho-p38 (*upper*), -total p38 (*middle*), and -HA (*lower*). Graphical presentations show the normalized density of phosphorylated JNK (*bottom* of *panel A*) and p38 (*bottom* of *panel B*), decaying from 100%.

buffered saline (pH 7.4)). Slides were rinsed with phosphate-buffered saline and incubated in permeabilization solution followed by TUNEL reaction mixture for 60 min at 37 °C in a humidified chamber. After a rinse, the slides were incubated with converter-alkaline phosphatase solution for 30 min at 37 °C and then detected with alkaline phosphatase substrate solution (Vector Laboratories, Burlingame, CA) for 10 min at 25 °C. After an additional rinse, the slides were mounted and analyzed under a light microscope. These experiments were performed in triplicate.

RESULTS

AKT2 Is Activated by UV Irradiation, Heat Shock, Hyperosmolarity, and TNFα—Previous studies showed that stress activates AKT1 and AKT3 but not AKT2 in fibroblasts (19). It has also been shown that TNFα receptor mediates UV- and heat shock-induced stress signaling (1–3). In agreement with these studies, we found that exposure of NIH 3T3 fibroblasts to UV-C, heat, or hyperosmotic conditions did not result in AKT2 activation (data not shown). It is possible, however, that stress might activate AKT2 in epithelial cells due to the fact of frequent alterations of AKT2, but not AKT1 and AKT3, in human epithelial tumors (7, 24, 27). For this reason we examined the effects of stress on AKT2 activation in two ovarian epithelial cancer cell lines, A2780 cells, which were transiently transfected with HA-AKT2, and OVCAR3 cells, which express high levels of endogenous AKT2 (7). The cells were exposed to UV-C, heat shock (45 °C), 0.4 M NaCl, or 20 ng/ml TNFα. IGF1-stimulated cells were used as controls. As assessed by *in vitro* kinase and Western blot analyses of AKT2 immunoprecipitates, all the stimuli substantially increased AKT2 activity in both A2780 and OVCAR3 cells (Figs. 1, *A* and *B*). The levels of AKT2 activity induced by these agents, however, were variable. AKT2 activity induced by TNFα and UV was comparable with that stimulated by IGF-1, whereas the effect of heat shock and hyperosmolarity (NaCl) on AKT2 activity was relatively smaller (Fig. 1). Nevertheless, these findings suggest that stresses activate AKT2 in a cell type-specific manner.

Stress Simulates PI3K That Mediates AKT2 Activation—To show that stress does indeed activate PI3K in epithelial cells, A2780 or HEK293 cells were exposed to UV irradiation, heat shock, and 0.4 M NaCl or TNFα, and cell lysates were immunoprecipitated with antibody to pan-p85, a regulatory subunit of PI3K. Assay of PI3K activity shows that these stress conditions as well as TNFα activated PI3K as efficiently as did IGF-1 (Fig. 2*A*). As described above, stress has been shown to activate AKT1 by both PI3K-dependent and -independent pathways

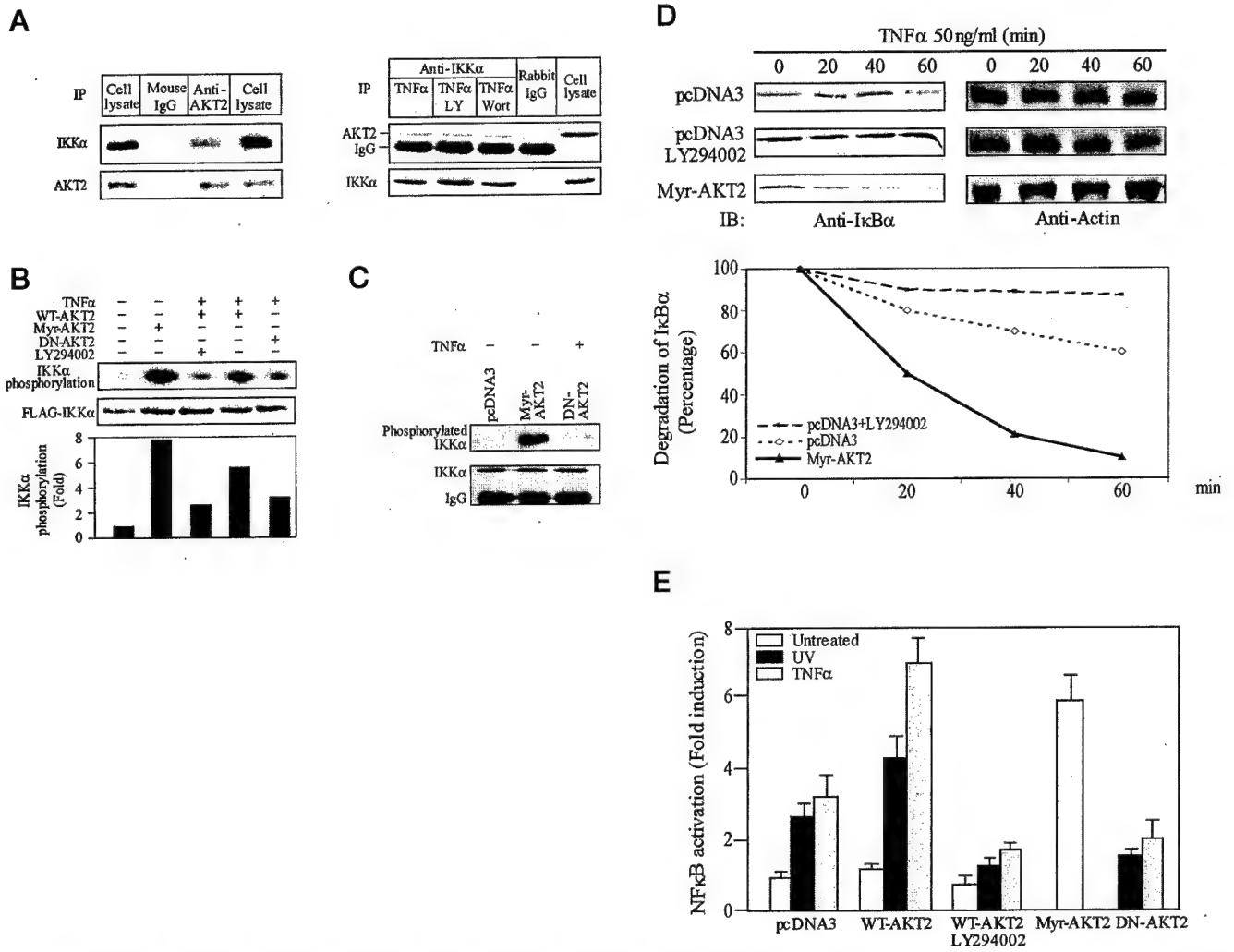


FIG. 4. **AKT2 interacts with and phosphorylates IKKα, leading to IκBα degradation and NFκB activation.** *A*, *left panel*, Western blotting analyses. HEK293 cell lysates were immunoprecipitated (*IP*) with anti-AKT2 or IgG (control) and detected with anti-IKKα (*top*) or anti-AKT2 (*bottom*) antibody. *Right panel*, HEK293 cells were treated with LY294002 (*LY*) or wortmannin for 30 min followed by TNFα for 20 min. Immunoprecipitates were prepared with anti-IKKα antibody or IgG and immunoblotted with antibody to AKT2 (*top*) or IKKα (*bottom*). *B*, *in vitro* kinase assay analyses of immunoprecipitates prepared from A2780 cells transfected with indicated plasmids using immunopurified FLAG-IKKα as substrates (*upper*). Expression of FLAG-IKKα was confirmed by immunoblotting analysis with anti-FLAG antibody (*middle*). The *bottom panel* shows the relative phosphorylation levels of IKKα by AKT2. *C*, *in vivo* labeling of IKKα from COS7 cells transfected with indicated DNA constructs treated with or without TNFα and incubated with [γ-^{32}P]orthophosphate for 4 h. IKKα immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, exposed to film (*top*), and then detected with anti-IKKα antibody (*bottom*). *D*, AKT2 induces IκBα degradation. HEK293 cells were transfected with indicated plasmids and treated with cycloheximide (50 μg/ml) for 1 h before treatment with 50 ng/ml TNFα for up to 60 min. Cell lysates were immunoblotted (*IB*) with antibody to IκBα (*left panels*) or β-actin (*right panels*). Degradation of IκBα was quantified with a densitometer. The *bottom panel* shows the degradation rate of IκBα by normalizing density of IκBα bands at 0 time point as 100%. *E*, reporter assays. HEK293 cells were transfected with 2×NFκB-Luc, β-galactosidase and WT-AKT2, Myr-AKT2, or DN-AKT2 pretreated with or without LY294002 and subsequently exposed to 40 J/m^2 UV-C or 20 ng/ml TNFα. Cell lysates were assayed for luciferase activity and normalized by β-galactosidase activity. *Error bars* represent S.D. Data were obtained from triplicate experiments.

(17, 18). To assess the role of PI3K in the stress-induced activation of AKT2, A2780 cells transfected with HA-AKT2 were exposed to 25 μM LY294002, a specific PI3K inhibitor, for 30 min before stress or TNFα treatments. LY294002 effectively inhibited stress- and TNFα-induced AKT2 activation (Fig. 2*B*). These data provide direct evidences of stress-induced activation of AKT2 through a PI3K-dependent pathway in human epithelial cells.

Stress-induced AKT2 Activation Inhibits UV- and TNFα-induced JNK and p38 Activities—Previous studies demonstrated that two groups of mitogen-activated protein kinases, the JNK and p38, are activated by environmental stress and TNFα (28). Therefore, we examined the effects of stress-induced AKT2 activation on the JNK and p38 to determine whether stressed-induced AKT2 activation could target these two stress kinases. A2780 cells were transfected with constitutively active AKT2 or pcDNA3 vector alone. Thirty-six hours after transfection, cells were treated with TNFα or UV and analyzed by Western blot for JNK and p38 activation using anti-phospho-JNK and anti-phospho-p38 antibodies. Both JNK and p38 were activated by TNFα and UV irradiation. The maximal activation was observed at 10 min of stimulation. Expression of constitutively active AKT2, however, exhibited inhibitory effects on the activation of JNK and p38 that was induced by TNFα and UV irradiation. Notably, the activation of JNK and p38 in constitutively active AKT2-transfected cells does not significantly differ from that of the cells transfected with pcDNA3 vector at 10 min of TNFα treatment. However, the phosphorylation levels of JNK and p38 in the cells expressing constitutively active AKT2 declined much more than that of

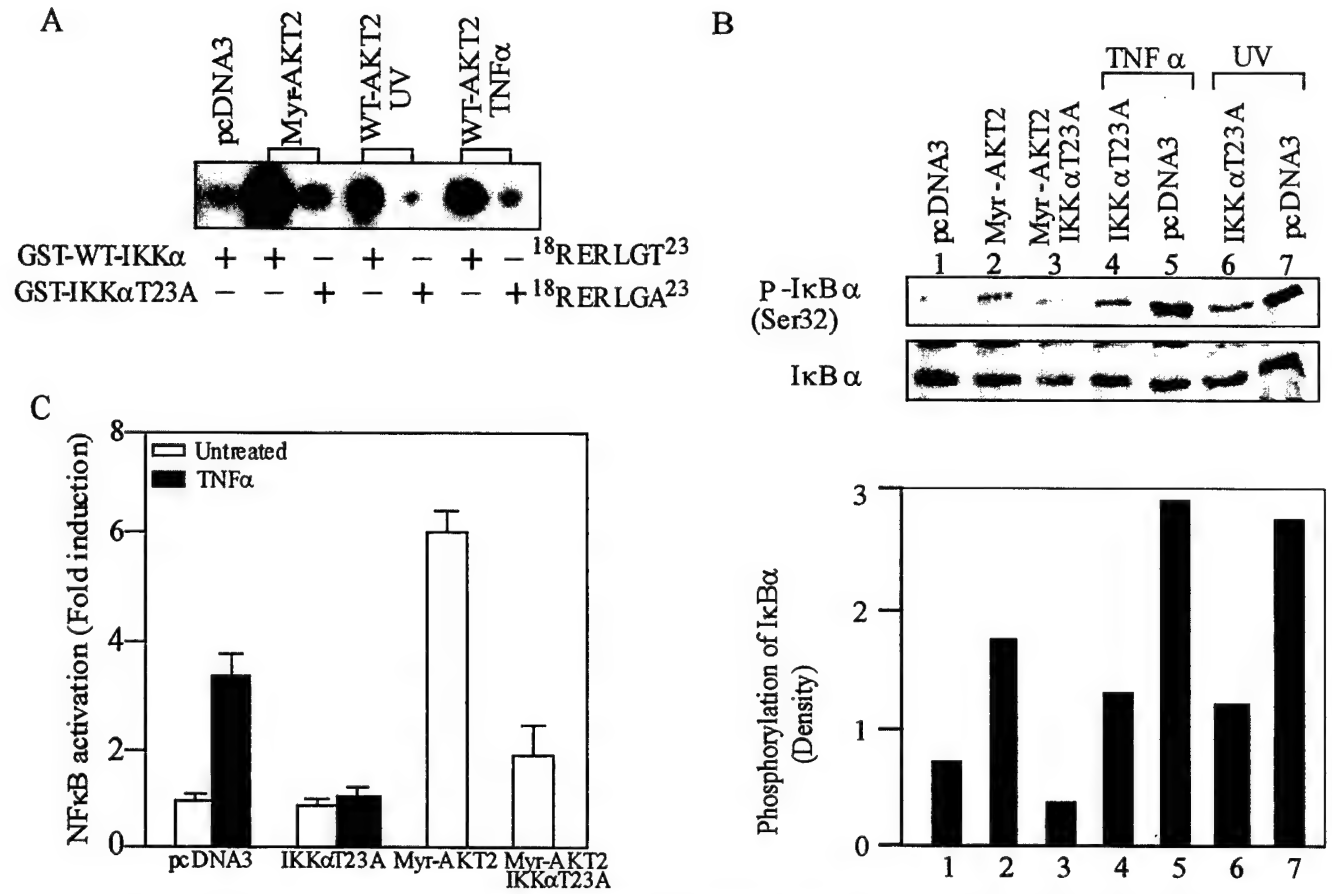


FIG. 5. **AKT2-phosphorylated IKKα at threonine 23 is required for stress-induced NFκB.** *A*, AKT2 phosphorylation of IKKα at threonine 23. *In vitro* kinase assay of AKT2 immunoprecipitates prepared from A2780 cells transfected with the indicated plasmids and treated with or without UV or TNFα. GST-fused wild type IKKα (**^{18}RERLGT^{23}**) or mutant IKKα (**^{18}RERLGA^{23}**) was used as the substrate. *B*, AKT2 induces IκBα phosphorylation (*P-*). HEK293 cells were transfected with the indicated expression constructs. Thirty-six hours after transfection, cells were treated with 20 ng/ml TNFα for 30 min or irradiated with 40 J/m^2 UV followed by a 30-min incubation. Cell lysates were immunoblotted with anti-phospho-IκBα (*upper*) or anti-IκBα (*middle*) antibody. The band density of the phospho-IκBα was quantified (*bottom*). *C*, luciferase reporter assay. HEK293 cells were transfected with the indicated plasmids. After treatment with or without 20 ng/ml TNFα for 12 h, cell lysates were assayed for luciferase activity and normalized by β-galactosidase activity. Results were obtained from three independent experiments.

pcDNA3-transfected cells after 30 min of stimulation (Fig. 3 and data not shown). We therefore conclude that the activation of AKT2 does not activate but rather inhibits TNFα- and UV-induced JNK and p38 activities.

AKT2 Interacts With and Phosphorylates IKKα, but Not NIK, Leading to IκBα Degradation and NFκB Activation—The capacity of both cellular stress and TNFα to activate the NFκB pathway is well documented (29). Previous studies also show that AKT1 induces activation of the NFκB by interaction with IKKα (13, 14). However, to date there are no reports addressing the potential role of AKT2 in the activation of the NFκB pathway. To determine whether AKT2 associates with IKKα, HEK293 cells were treated with or without TNFα, immunoprecipitated with anti-AKT2, and immunoblotted with anti-IKKα antibody or vice versa. In both instances, the association of AKT2 with IKKα was observed (Fig. 4*A*). Additional studies showed that AKT2-IKKα interaction was unaffected by treatment of cells with PI3K inhibitor, wortmannin, or LY294002 (Fig. 4*A*). These findings indicate that AKT2 constitutively associates with IKKα. In addition, we have identified putative AKT2 phosphorylation sites in the IKKα (**^{18}RERLGT^{23}**) and in NFκB-inducing kinase (NIK, **^{366}RSREPS^{371}**) (bold residue letters represent Akt consensus sequence). To determine whether IKKα and/or NIK are phosphorylated by AKT2, A2780 cells were transfected with different forms of AKT2 and treated with LY294002 and TNFα. *In vitro* AKT2 kinase assays were performed using FLAG-IKKα or HA-NIK, purified from the transfected COS7 cells, as substrate. Repeated experiments show that TNFα-induced AKT2 and constitutively active AKT2 phosphorylated IKKα (Fig. 4*B*) but not NIK (data not shown). Phosphorylation of IKKα induced by TNFα was largely attenuated by PI3K inhibitor LY294002. Quantification analyses revealed that approximate 70% of TNFα-induced IKKα phosphorylation was inhibited by pretreatment with LY294002 (Fig. 4*B*). Furthermore, we assessed AKT2 to determine if it phosphorylates IKKα *in vivo*. COS7 cells were transfected with FLAG-IKKα together with either constitutively active or dominant-negative AKT2 or vector alone and labeled with [γ-^{32}P]orthophosphate. IKKα immunoprecipitates prepared using anti-FLAG antibody were separated by SDS-PAGE and transferred to nitrocellulose. The phospho-IKKα was detected by autoradiography. As shown in Fig. 4*C*, IKKα was highly phosphorylated in cells expressing constitutively active AKT2 but not in the cells transfected with pcDNA3 and dominant-negative AKT2. Collectively, these data indicate that IKKα is an AKT2 physiological substrate.

Activation of NFκB requires its dissociation from its cytosolic inhibitor, IκB, a process dependent on the phosphorylation and consequent degradation of IκB by IKK. Thus, we next examined AKT2 to determine if it induces IκB degradation. Immunoblotting analyses revealed that constitutively active AKT2 significantly promoted IκBα degradation (Fig. 4*D*). To assess the involvement of AKT2 in NFκB activation, HEK293 cells were co-transfected with a NFκB-luciferase reporter and either

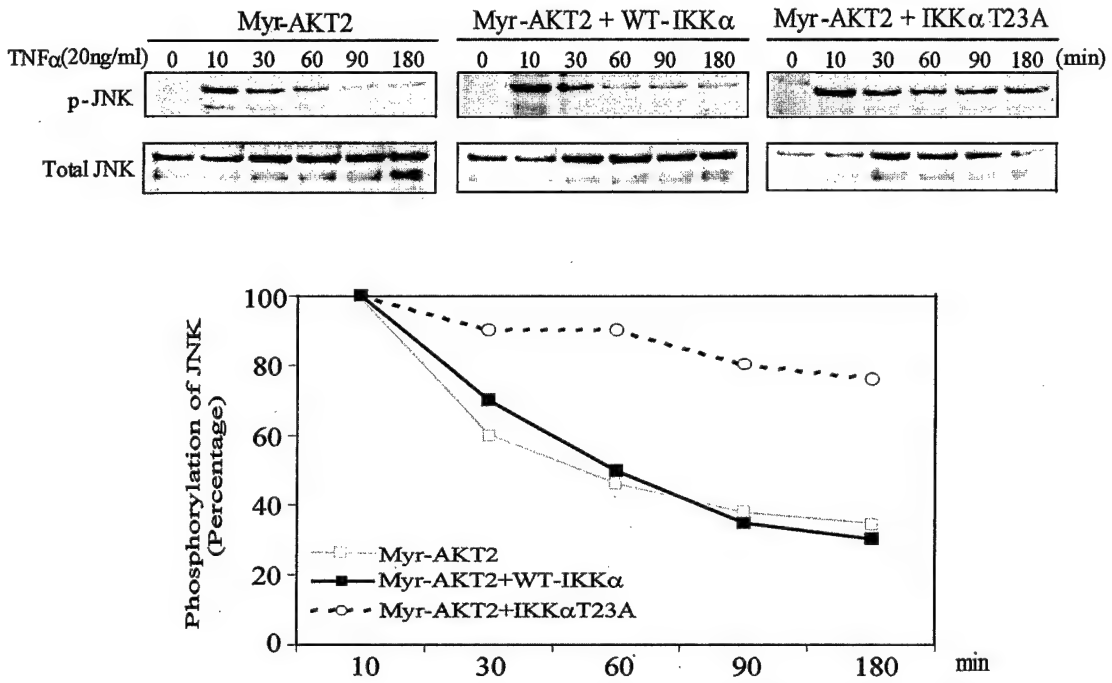


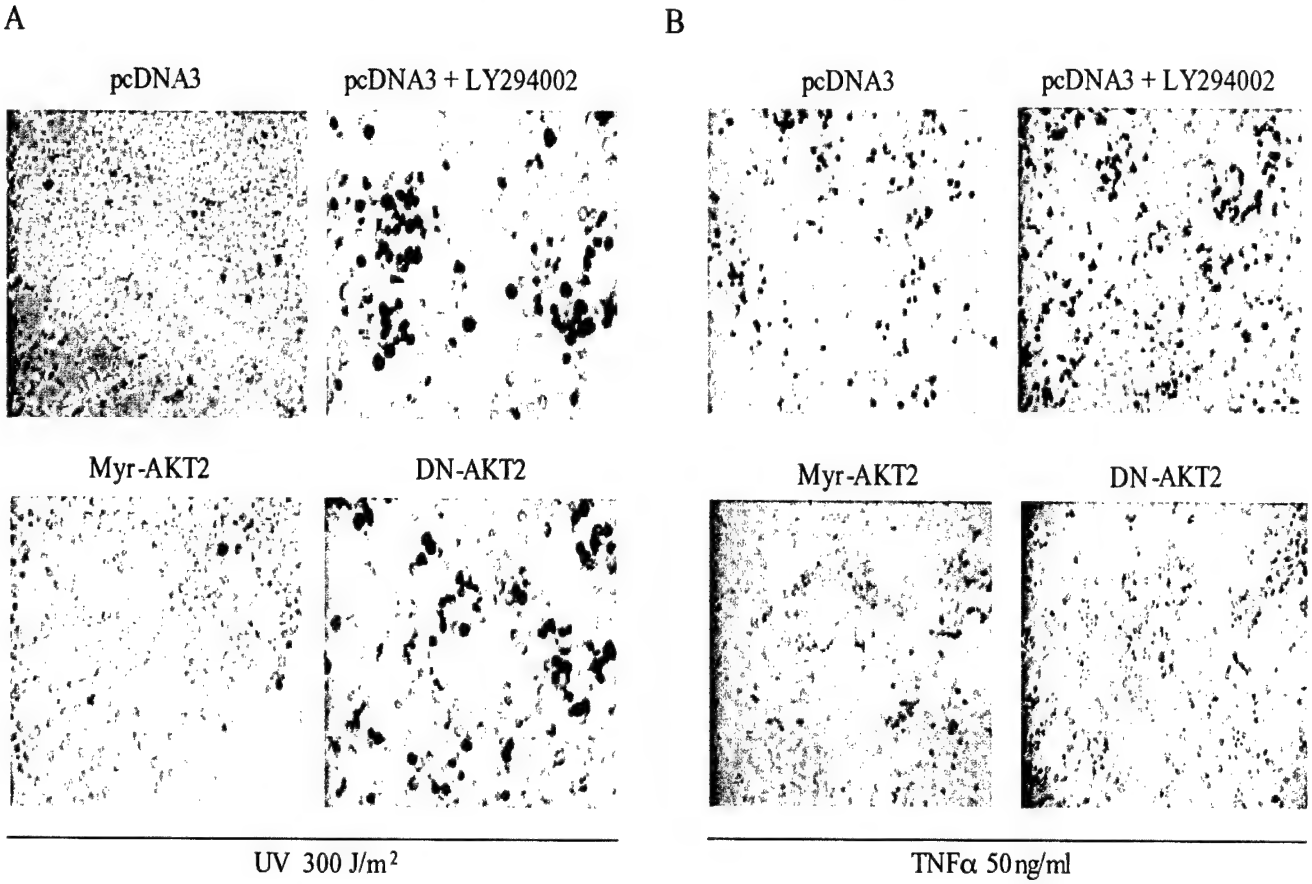
FIG. 6. **AKT2 phosphorylation of IKKα is required for inhibition of TNFα-induced JNK activity.** Immunoblotting analyses of HEK293 cells transfected with indicated expression constructs and treated with TNFα (20 ng/ml). The blots were probed with anti-phospho-JNK (*p-JNK*; *upper*) and -total JNK (*middle*) antibodies. Results represent one of three independent experiments. The *bottom panel* shows the quantification of phosphorylated JNK at the indicated time points.

vector alone, wild type, or constitutively active or dominant negative AKT2 treated with or without LY294002 before UV or TNFα stimulation. As shown in Fig. 4*E*, ectopic expression of wild-type AKT2 significantly enhanced UV- and TNFα-induced NFκB activity, which was abolished by treatment of cells with LY294002 or dominant negative AKT2. Constitutively active AKT2 alone was able to induce NFκB activity to a level comparable with UV- or TNFα-treated cells transfected with wild-type AKT2. These data show that PI3K/AKT2 mediates both stress- and TNFα-activated NFκB pathway.

To determine AKT2 phosphorylation site of IKKα, GST fusion proteins containing either wild type IKKα (^{18}**RERLGT**^{23}; termed GST-WT-IKKα) or mutant IKKα (^{18}**RERLGA**^{23}; termed GST-IKKαT23A) were prepared and used as substrates in *in vitro* AKT2 kinase assays. As seen in Fig. 5*A*, UV- and TNFα-activated AKT2 as well as constitutively active AKT2 phosphorylated GST-WT-IKKα but not GST-IKKαT23A. We next assessed the capacity of AKT2-induced IKKα to phosphorylate IκBα. Constitutively active AKT2 was expressed in HEK293 cells, and cell lysates were immunoblotted with an antibody that specifically recognizes phosphorylated IκBα at Ser^{32}. The results of these experiments show that constitutively active AKT2 increased IκBα phosphorylation ~2-fold and that this increase was abolished by cotransfection of pcDNA3-IKKαT23A. Expression of IKKαT23A also blocked IκBα phosphorylation induced by TNFα or UV (Fig. 5*B*). Additional luciferase reporter experiments demonstrated that expression of IKKαT23A inhibited the TNFα- or constitutively active AKT2-induced NFκB activation (Fig. 5*C*). These data indicate that phosphorylation of IKKα at Thr^{23} is required for AKT2-mediated NFκB activation.

IKKα Phosphorylation by AKT2 Is Required for Inhibition of JNK but Not p38 Activation—Recent studies showed that NFκB exerts its cell survival function by inhibition of JNK activation in response to extracellular stress (30, 31). However, it is currently unknown whether AKT-induced NFκB activation results in inhibition of JNK. Therefore, we next attempted to determine if AKT2-activated IKKα is required for AKT2 inhibition of JNK and p38 activities induced by stress and TNFα. The activation of JNK and p38 was examined in HEK293 cells transfected with IKKα or IKKαT23A together with or without constitutively active AKT2. Western blotting analyses with phospho-JNK and -p38 antibodies revealed that wild type IKKα did not significantly enhance AKT2 inhibition of JNK (Fig. 6). However, expression of IKKαT23A abrogated the effects of constitutively active AKT2 on inhibition of JNK (Fig. 6). Similar to the results shown in Fig. 3, TNFα-induced JNK activation reached the maximal level at 10 min of stimulation, which was neither significantly inhibited by constitutively active AKT2 nor affected by expression of IKKαT23A (Fig. 6). Therefore, these data indicate that inhibition of JNK activation by AKT2/NFκB could be via a mechanism of induction of dephosphorylation of JNK by the AKT2/IKKα/NFκB cascade.

AKT2 Activation Inhibits Stress-induced Apoptosis—It is documented that various stresses and TNFα are capable of inducing apoptosis in different cell types through activation of JNK and p38 pathways (29). Because PI3K/AKT is essential for cell survival and activated AKT2 inhibits JNK/p38 and induces NFκB pathway, we investigated the role of PI3K/AKT2 in stress- and TNFα-induced programmed cell death. AKT2 stably transfected A2780 cells were pretreated with or without LY294002 for 2 h before exposure to UV, heat shock, NaCl, or TNFα. As determined by the TUNEL assay, inhibition of PI3K activity dramatically increased the percentage of cells undergoing apoptosis in response to UV or TNFα (Fig. 7). Moreover, inhibition of AKT2 activity by expression of dominant-negative AKT2 increased the percentage of apoptotic cells in the UV- and TNFα-treated populations by ~2-fold. On the other hand, cells expressing constitutively active AKT2 were resistant to UV- and TNFα-induced apoptosis. These data show that the PI3K/AKT2 pathway plays a key role in protecting cells from apoptosis induced by extracellular stress or TNFα.



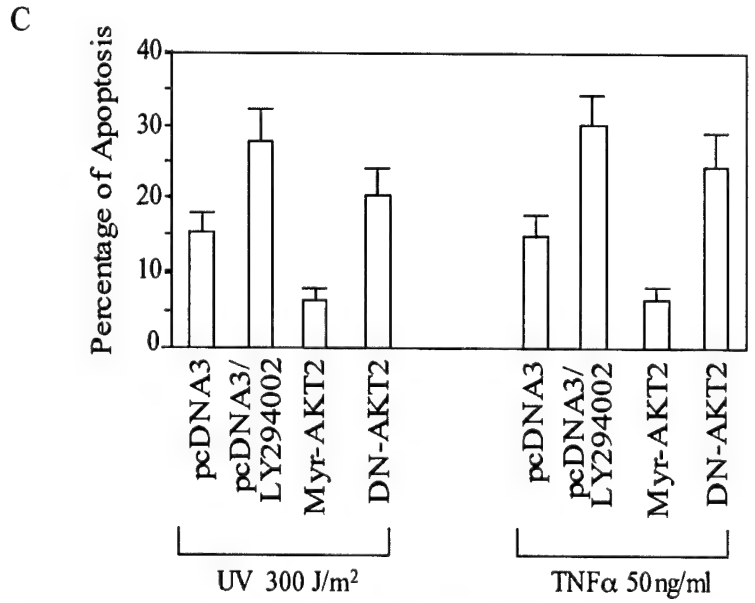


FIG. 7. **AKT2 activation inhibits stress-induced apoptosis.** A2780 cells stably transfected with constitutively active AKT2, DN-AKT2, or vector alone were pretreated with or without 25 μM LY294002 for 2 h before exposure to UV-C (300 J/m²) (*A*) or TNFα (50 ng/ml) for 24 h (*B*). Apoptosis was assessed by TUNEL assay. *C*, quantitation of data shown in *A* and *B* were derived from triplicate experiments. *Error bars* represent S.D.

DISCUSSION

In this report, we have provided evidence that AKT2 is activated by extracellular stress and TNFα through a PI3K-dependent pathway in human epithelial cells. Most importantly, the activation of AKT2 inhibits stress- and TNFα-induced JNK and p38 activities and activates the NFκB cascade, leading to protection of cells from stress- and TNFα-induced apoptosis.

Previous studies show that stress activates cell membrane

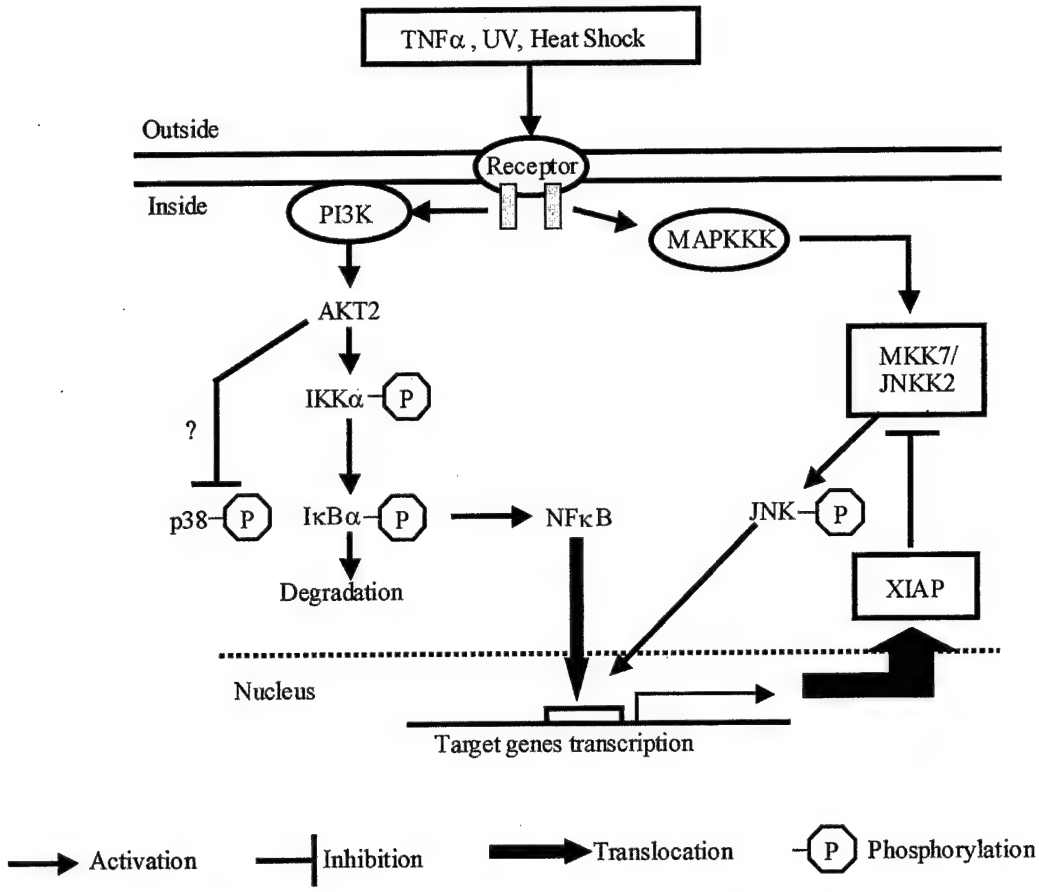


FIG. 8. **Schematic illustration of negative regulation of JNK by AKT2/NFκB.**

receptors, including those for epidermal growth factor, platelet-derived growth factor, and IGF. As a result, receptors associate with numerous proteins that activate downstream signaling molecules (1–3). One such protein is PI3K, which has been implicated in the regulation of nearly all stress signaling pathways (1). Because the AKTs are major downstream targets of PI3K, their role in the stress response has been recently investigated. In Swiss 3T3 cells, both oxidative stress and heat shock were shown to induce a marked activation of AKT1 and AKT3 but not AKT2 (19). AKT1 activation by hyperosmotic stress in COS7 and NIH 3T3 cells has also been demonstrated (17). In this study, we show that AKT2 is activated by different stress conditions including UV irradiation, hyperosmolarity, and heat shock as well as by TNFα in several human epithelial cell lines.

Three isoforms of AKT display high sequence homology and share similar upstream regulators and downstream targets as identified so far. However, there are clear differences between them in terms of biological and physiological function. In addition to the more prominent role of AKT2 in human malignancy and transformation (7, 32), the expression patterns of AKT1, AKT2, and AKT3 in normal adult tissues as well as during development are quite different (4, 8, 33). Recent studies suggest that AKT1, AKT2, and AKT3 may interact with different proteins and, thus, may play different roles in signal transduction. For instance, the Tcl1 oncoprotein preferentially binds to and activates AKT1 but not AKT2 (34). Gene knockout studies revealed that AKT1-deficient mice display defects in both fetal and postnatal growth but, unlike $AKT2^{-/-}$ mice, do not exhibit a type II diabetic phenotype; these differences suggest that the functions of AKT1 and AKT2 are non-redundant with respect to organismic growth and insulin-regulated glucose metabolism (21–23). It has been also shown that AKT2 but not AKT1 plays a specific role in muscle differentiation (35).[2] In this study, we demonstrated that AKT2 is activated by a variety of stress conditions in human epithelial cells but not in fibroblasts, suggesting that activation of different isoforms of AKT is cell type-specific in response to extracellular stress.

It is controversial whether stress-induced AKT1 activation is mediated by the PI3K pathway (17–19). Two previous reports showed that PI3K inhibitors did not block heat shock- or H_2O_2-induced activation of AKT1 and, thus, suggested that stress (unlike growth factors) activates AKT1 in a PI3K-indpendent manner (17, 18). However, the opposite results were observed by other groups (19, 20). Konishi *et al.* also provide evidence of AKT1 activation by H_2O_2 and heat shock through both PI3K-dependent and -independent pathways (18). We previously demonstrated that activation of AKT2 by growth factors required PI3K activity, whereas both PI3K-dependent and -independent pathways contributed to AKT2 activation by Ras (26). In this report, we show that PI3K inhibitors completely block AKT2 activation induced by UV-C, heat shock, and hyperosmolarity, indicating that stress activates AKT2 via the PI3K pathway.

JNK and p38 are stress mitogen-activated protein kinases that are activated by cytokines and a variety of cellular stresses (28). Like the classical mitogen-activated protein kinase kinase (MEK), direct activators for JNK and p38 have been identified. JNK is activated by phosphorylation of tyrosine and threonine by the dual specificity kinases, MKK4/SEK1 and MKK7. Similarly, p38 is activated by MKK3 and MKK6. However, biochemical studies have documented the existence of other JNK

[2] S. Kaneko, S. V. Nicosia, Z. Wu, T. Nobori, and J. Q. Cheng, submitted for publication.

and p38 activators or inhibitors in cells stimulated by a variety of cellular stresses (28). Although previous reports showed that AKT, JNK, and p38 are downstream targets of PI3K and represent parallel pathways in response to stress (17–20, 37, 38), the data presented in this study indicate that stress- and TNFα-induced activation of AKT2 inhibits the JNK and p38 activities, suggesting that AKT2 cross-talks with JNK and p38 stress pathways.

NFκB is another critical stress response pathway (29). Activation of NFκB is achieved through the signal-induced proteolytic degradation of IκB, which is associated with and inhibits the activity of NFκB in the cytoplasm. The critical event that initiates IκB degradation is the stimulus-dependent activation of the IκB kinases IKKα and IKKβ, which phosphorylate IκB at specific N-terminal serine residues (Ser^{32} and Ser^{36} for IκBα; Ser^{19} and Ser^{23} for IκBβ). Phosphorylated IκB is then selectively ubiquitinated by an E3 ubiquitin ligase and degraded by the 26 S proteasome, thereby releasing NFκB for translocation to the nucleus where it initiates the transcription of target genes (29). Moreover, two mitogen-activated protein kinase kinase kinase (MAPKKK) members, NIK and MEKK1, have been reported to enhance the activity of the IKKs and consequently trigger the phosphorylation and destruction of the IκBs and induce the activation of the NFκB pathway (29). Recent studies also showed that AKT1 induces the NFκB cascade through activation of IKK and degradation of IκB (13, 14). In this report, we show that AKT2 physically binds to and phosphorylates IKKα but not NIK even though NIK contains an AKT2 phosphorylation consensus sequence. When activated by stress or TNFα, AKT2 degrades IκB and activates NFκB-mediated transcription, indicating that stress-activated AKT2 targets the NFκB pathway.

Importantly, we have provided evidence that activation of AKT2 induced by stress and TNFα inhibits JNK activity through activation of the NFκB pathway to protect cells from apoptosis in response to these stimuli. Previous studies showed that the AKT2 pathway is important for cell survival and malignant transformation (7, 24, 32). The data presented here show that cells expressing constitutively active AKT2 are resistant to stress- and TNFα-induced apoptosis and that dominant-negative AKT2 and LY294002 sensitize cells to stress- and TNFα-induced programmed cell death. These findings indicate that stress-induced AKT2 activation promotes cell survival. Among the stress-activated kinases are JNK; recent studies demonstrated that activation of JNK and p38 plays an important role in triggering apoptosis in response to extracellular stress and TNFα (36, 39–41), whereas activation of NFκB protects cells from programmed cell death (29). Although a number of downstream targets of AKT2 have been identified, our data indicate that AKT2-inhibited JNK and p38 activities and AKT2-induced NFκB activation could play, at least in part, an important role in the AKT2 pathway that protects cells from stress- and TNFα-induced apoptosis. Recent reports demonstrate that NFκB-up-regulated Gadd45β and Xiap inhibited JNK activation and abrogated TNFα-induced programmed cell death (30, 31). Our cDNA microarray experiments showed that constitutively active AKT2 induces *Xiap*.[3] Thus, AKT2 inhibition of JNK activity could be due to up-regulation of Xiap by NFκB pathway (Fig. 8). Further studies are required to characterize the mechanism of inhibition of p38 stress pathway by AKT2 and involvement of Xiap in AKT2/NFκB inhibition of the JNK activation.

[3] M. Sun and J. Q. Cheng, unpublished data.

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Drug Resistance Updates 5 (2002) 131–146

DRUG RESISTANCE *Updates*

Review

Role of X-linked inhibitor of apoptosis protein in chemoresistance in ovarian cancer: possible involvement of the phosphoinositide-3 kinase/Akt pathway

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Abstract

Although cisplatin derivatives are first-line chemotherapeutic agents for the treatment of epithelial ovarian cancer, chemoresistance remains a major hurdle to successful therapy and the molecular mechanisms involved are poorly understood. Apoptosis is the cellular underpinning of cisplatin-induced cell death, which is associated with expression of specific "death" genes and down-regulation of "survival" counterparts. The X-linked inhibitor of apoptosis proteins (Xiap), an intracellular anti-apoptotic protein, plays a key role in cell survival by modulating death signaling pathways and is a determinant of cisplatin resistance in ovarian cancer cells in vitro. This review focuses on the role of Xiap and its interactions with the phosphoinositide-3 kinase (PI3K)/Akt cell survival pathway in conferring resistance of ovarian cancer cells to chemotherapeutic agents and discusses potential therapeutic strategies in overcoming chemoresistant ovarian cancer.

Keywords: Xiap; PI3K/Akt; Ovarian cancer; Chemoresistance

1. Introduction

Ovarian cancer is the leading cause of cancer death in women in the Western world and ranks fifth among the most common female cancers. The lifetime risk of developing ovarian cancer is 1 in 70 and a lifetime chance of dying of ovarian cancer is 1% (Reis et al., 2000; American Cancer Society, 1997). Epithelial ovarian cancer constitutes approximately 90% of all human ovarian malignancies and originates from the simple epithelium covering the surface of the ovary (Auersbergs et al., 2001; Eltabbakh and Awtrey, 2001). Due to the tendency of most malignant ovarian tumors to insidiously spread within the abdominal cavity by seeding, they are often detected at advanced stages of the disease for which definite cure rates are often low. Indeed, only 25% of patients diagnosed with ovarian cancer have the disease localized in the ovary and the cure rate for advanced stages of cancers (stages III and IV) is below 30%. While over 50% of all ovarian cancer patients will now survive 5 years, cure rates have remained basically the same over the last 20 years (Reis et al., 2000; American Cancer Society, 1997).

Cisplatin and its analogues have been most frequently used for treatment of human cancer, including ovarian cancer. While adjuvant chemotherapy with paclitaxel and cisplatin or carboplatin achieves clinical response in approximately 80% of patients, the tumor recurs in most patients within 3 years following treatment (Eltabbakh and Awtrey, 2001). The overall 5-year survival rate for advanced ovarian cancer patients is still low (20–30%) and is due to chemoresistance in the primary or recurrent tumors, thus resulting in treatment failure. Similarly, although ovarian cancer patients show high response rates to initial chemotherapy after cytoreductive surgery, most patients also develop resistance to chemotherapy during treatment (Katsaros et al., 1999). Overcoming drug resistance is the

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key to successful treatment of ovarian cancer (Kikuchi, 2001).

While the clinical and histological prognostic factors (e.g. surgical stage and tumor grade) are well understood, less is known about the biological process leading to uncontrolled cellular growth. It is now widely accepted that tissue growth is not only dependent on cell proliferation, but also on the rate of apoptosis. Imbalance between these processes leads to uncontrolled tissue growth (Kerr et al., 1994; Reed, 1994; Steward, 1994; Thompson, 1995). The progression of the tumor is, in part, due to the failure of the cells to undergo apoptosis in response to a death signal (Naik et al., 1996). Thus, it is generally accepted that carcinomas may be caused or promoted by factors inhibiting cell death as well as those enhancing cell survival. Whereas the action of cisplatin is thought to be associated with its ability to form inter- and intra-strand DNA cross-links (Li et al., 2000), recent reports suggest that apoptosis may be the cellular underpinning of cisplatin-induced cell death (Li et al., 2000; Sasaki et al., 1999; Arts et al., 2000) and that the DNA-damaging effects of cisplatin are also associated with expression of specific death genes and down-regulation of "survival" counterparts (Li et al., 2000, 2001; Sasaki et al., 1999; Arts et al., 2000; Schneiderman et al., 1999).

The molecular mechanism of chemoresistance in ovarian cancer is multifactorial and poorly understood. It is recognized that 75% of patients resistant to cisplatin treatment also developed resistance to taxol, implying that the resistance to these agents may involve similar mechanisms. Chemoresistance may generally be classified into acquired and de novo forms and has traditionally been thought of in terms of altered drug transport and gene expression (e.g. multi-drug resistance gene), modified drug target, increased rate of DNA repair or decreased rate of drug-induced DNA or macromolecule damage (Reed et al., 1996). A growing body of evidence indicates that defects in the intra- and extra-cellular apoptotic pathways are an important cause of resistance to cytotoxic agents (Arts et al., 2000). Indeed, our recent studies indicate that homeostasis of human ovarian surface epithelial cells is maintained by a delicate balance in the expression and actions of tumor suppressors (e.g. p53, Fas and FasL) and cell survival factors (e.g. inhibitor of apoptosis proteins (IAPs) and intermediates of the PI3K/Akt cell survival pathway). IAPs are suppressors of apoptosis and believed to be determinants of chemosensitivity of ovarian cancer. Moreover, the intermediates of the PI3K/Akt survival pathway are frequently altered in human ovarian cancer (Shayesteh et al., 1999; Cheng et al., 1992; Yuan et al., 2000; Sun et al., 2001a; Philp et al., 2001) and elevated levels of PI3K and AKTs were shown to be associated with poor prognosis and chemoresistance in this malignancy (Sun et al., 2001a; Philp et al., 2001; Page et al., 2000). Chemoresistance in ovarian cancer results in part from over-expression of IAPs, PI3K, and/or AKTs and down-regulation of Fas and FasL, leading to suppressed apoptosis (Li et al., 2000, 2001; Sasaki et al., 1999; Schneiderman et al., 1999).

2. Xiap: a determinant in chemosensitivity in ovarian cancer

The progression of the tumor is, in part, due to suppressed apoptosis (Naik et al., 1996). It has been shown that environmental stress (e.g. DNA damage, drug-induced cell-cycle arrest) elicits rapid cellular responses (e.g. expression of oncogenes and DNA-repair enzymes) for homeostatic maintenance. Thus, it is conceivable that some "cell survival" factors (cell death inhibitors) may be induced and play a promoting role in this process. While expression of *bcl-2* is an important factor in drug-induced apoptosis in ovarian cancer thereby contributing to chemoresistance (Eliopoulos et al., 1995; Herod et al., 1996), evidence also indicates that *bcl-2* over-expression cannot adequately account for the etiology of drug resistance in ovarian cancer (Steward, 1994; Diebold et al., 1996). The IAPs are potential candidates involved in the suppression of apoptosis and in ovarian cancer progression.

The IAPs are intracellular anti-apoptotic proteins, first identified in baculovirus, which play a key role in cell survival by modulating death-signaling pathways. They currently include X-linked IAP (Xiap), human IAP-1 (Hiap-1), human IAP-2 (Hiap-2), neuronal apoptosis inhibitory protein (Naip), survivin and Livin (Fig. 1) (Ambrosini et al., 1997; Liston et al., 1996; Kasof and Gomes, 2001; Lin et al., 2000). These proteins are characterized by the presence of a caspase-recruitment domain (CARD) and an N-terminal baculovirus-inhibitor-of-apoptosis-repeat (BIR) motif, which is necessary for biological activity. With the exception of Naip and survivin, IAPs also contain a C-terminal RING-Zinc finger domain believed to be involved in protein–protein and protein–nucleic acid interactions. Importantly, a recent study reported that the RING finger domain of Xiap and Hiap-1 has ubiquitin protease ligase (E3) activity and is responsible for the autoubiquitination and degradation of IAPs after apoptosis stimulus (Yang et al.,

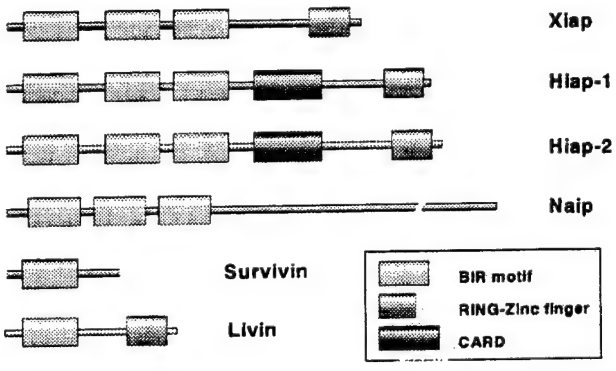


Fig. 1. Diagrammatic representation of the structure of mammalian IAP family members. All IAPs have one or more baculovirus inhibit or of apoptosis repeat (BIR) motifs required for biological activity. Apart from Naip and Survivin, they have RING Zing finger involved in protein–protein interactions and auto-ubiquitination. Hiap-1 and Hiap-2 also have caspase recruitment domain (CARD).

2000). Only a few reports to date have addressed the mechanisms of action of these anti-apoptotic proteins. Hiap-1 and Hiap-2 have been suggested to suppress TNF receptor signaling by binding to TNF-receptor-associated factor. Xiap is a direct inhibitor of caspase-3 and caspase-7 and modulates the Bax/cytochrome c pathway by inhibiting caspase-9 (Takahash et al., 1998; Deveraux et al., 1999, 1998). The anti-apoptosis effects of mammalian IAPs have been well demonstrated, although to different extent depending on the specific apoptosis inducer, cell type and IAP examined (Liston et al., 1996; Uren et al., 1996; Duckett et al., 1996).

The role of Xiap in the regulation of apoptosis in human ovarian cancer has recently been examined. Xiap has been localized in human ovarian carcinomas, with levels being highest in proliferative but not apoptotic epithelial cells (Li et al., 2001). In contrast, expression of Hiap-1, Hiap-2 and Naip is relatively low in these cells. In addition, in vitro studies with cisplatin-sensitive ovarian cancer cell lines (OV2008 and A2780s) and their resistant variants (C13 (with wild type p53) and A2780cp (with mutated p53), respectively) (Liu et al., 1994; Delmastro et al., 1997; Behrens et al., 1987; Andrews et al., 1992) have shown that cisplatin treatment induced a rapid (within 3 h) increase in the Xiap protein content but decreased the Xiap level and induced apoptosis in sensitive cells but not their resistant variants with continuous drug challenge (Figs. 2A and B, Li et al., 2001; Asselin et al., 2001). The differences in the apoptotic response of these cell lines to cisplatin could not be attributed to the relative levels of cisplatin retention (Zinkewich-Peotti and Andrews, 1992). Direct evidence for an important role of Xiap in the regulation of apoptosis in ovarian cancer cells came from experiments in which down-regulation of Xiap by adenoviral Xiap antisense cDNA expression in the cisplatin-sensitive cells in the absence of the chemotherapeutic agent, induced caspase-3 and -9 cleavage and apoptosis (Li et al., 2001; Asselin et al., 2001). More importantly, cisplatin-induced apoptosis in cisplatin-sensitive cells was suppressed by adenoviral Xiap sense expression. These studies strongly suggest that Xiap is a determinant of cisplatin-sensitivity in human ovarian cancer cells and that cisplatin-resistance in human ovarian cancer may in part be due to an inability of this chemotherapeutic agent to decrease Xiap protein content and induce apoptosis (Li et al., 2000, 2001; Asselin et al., 2001). This concept is supported by recent observations on the anti-apoptotic role of Xiap in an oral carcinoma cell line (Matsumiya et al., 2001).

The mechanism by which cisplatin decreases Xiap level in ovarian cancer cells is not fully understood. We have recently shown that cisplatin can decrease Xiap protein content in chemosensitive ovarian cancer cells without a significant change in Xiap mRNA abundance (Asselin et al., 2001), suggesting that *Xiap* gene transcription is not involved in Xiap down-regulation by cisplatin. This notion is consistent with the recent finding that Xiap in thymocytes is degraded in a proteasome-dependent manner in response to the pro-apoptotic action of glucocorticoids or etoposide. Furthermore, Xiap has ubiquitin ligase (E3) activity at the zinc RING finger domain and is able to catalyze its own ubiquitination (Yang et al., 2000). In this regard, over-expression of a zinc RING finger domain deleted-Xiap is more effective than of the wild-type Xiap in preventing cisplatin-induced apoptosis in a cisplatin-sensitive ovarian cancer line (Sasaki et al., 2002) and removal of the RING domain of *Drosophila* IAPs enhances their ability to inhibit cell death caused by expression of the cell death-inducing protein Reaper (Yang et al., 2000). These findings raise the interesting possibility that Xiap auto-ubiquitination and degradation may be an important mechanism which regulates the steady-state Xiap level in ovarian cancer cells and determines the sensitivity of the cells to the pro-apoptotic action of cisplatin.

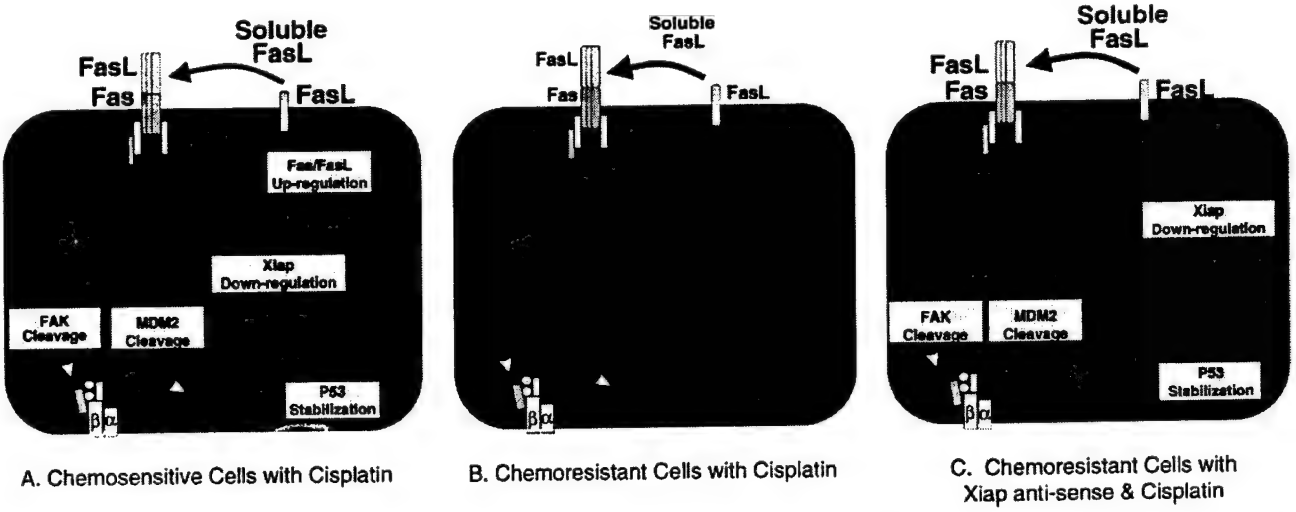


Fig. 2. Models showing the influence of cisplatin and Xiap anti-sense on chemosensitive and chemoresistant ovarian cancer cells. Xiap maintains cell survival by suppressing caspase-3 activation. As observed in chemosensitive cells treated with cisplatin, Xiap down-regulation in chemoresistant cells by anti-sense expression results in caspase-3 activation, FAK and MDM2 cleavage, increased p53 levels (via stabilization) and apoptosis.

3. Down-regulation of Xiap induces apoptosis and increases cisplatin-sensitvity

Xiap is often considered a final guardian in preventing and regulating apoptotic cell death. Thus, removal of the anti-apoptotic factor would be expected to result in unleashing the apoptotic process, probably mediated by the release of caspase-3 from its inhibition. Indeed, Xiap down-regulation following adenoviral Xiap antisense expression alone resulted in a concentration- and time-dependent decrease in Xiap content and an increase in apoptosis in the wild-type p53, cisplatin-resistant ovarian cancer cell line (C13) as well as it sensitized the cells to the cytotoxic action of cisplatin. The onset of apoptosis following Xiap down-regulation was associated with detectable cleavage of caspase-3 (activation) and MDM2, an oncoprotein that binds to and facilitates ubiquitin-mediated degradation of p53, as well as a significant p53 accumulation (Fig. 2C, Sasaki et al., 2000). In vitro MDM2 cleavage analysis also indicated that the oncoprotein is a substrate for caspase-3. These findings are consistent with the concept that caspase-3 activation following cisplatin challenge results in MDM2 degradation and p53 stabilization and eventually increase in p53 levels and induction of p53-mediated apoptosis (Fig. 2A, Sasaki et al., 2000).

It is of interest to note that the apoptotic response to Xiap down-regulation was not observed in p53-mutated (A2780-cp) or null (SKOV3) cell lines. While wild-type p53 expression in these genetically deficient cells led to significant apoptosis, co-expression of Xiap antisense and wild type p53 sense in these cells not only resulted in a higher p53 content but also was more effective in eliciting the apoptotic response than wild-type p53 restoration alone (Sasaki et al., 2000). These results are corroborated by the finding that the responsiveness of ovarian cancer to cisplatin is dependent on the normal function of p53 (Laframboise et al., 2000). Taken together, these observations provide strong support for a central role for Xiap in the control of p53 accumulation in ovarian cancer cells, a phenomenon associated with the inhibitory action of Xiap on caspase-3 activity, and provide a framework for novel therapeutic approaches in the treatment of chemoresistant ovarian cancer.

Recent reports have shown that IAPs may play an important role in the control of both receptor-mediated cell survival and death signaling by directly inhibiting downstream caspases (Sasaki et al., 2002; Roy et al., 1997; Deveraux et al., 1997). Several DNA damaging agents, including cisplatin, can up-regulate the expression of Fas (a 45 kDa cysteine-rich transmembrane glycoprotein belonging to the TNF/NGF receptor super-family (Itoh et al., 1991)) and its ligand (FasL) in a variety of tumor cell types including human ovarian cancer cells (Uslu et al., 1996; Muller et al., 1997; Schneiderman et al., 1999). Activation of Fas following Fas ligation leads to the initiation of the apoptotic process, which involves the activation of caspase-8 and caspase-3. While cisplatin is effective in up-regulating Fas expression in cisplatin-sensitive (OV2008 and A2780-s) and resistant (C13) ovarian cancer cell lines and activation of the Fas receptor with a monoclonal activating anti-Fas antibody following the cisplatin challenge induces apoptosis, the apoptotic response in the resistant cells were considerably lower compared to its sensitive counterpart (Schneiderman et al., 1999). Interestingly, cisplatin was effective in inducing the cleavage (activation) of caspase-8 and -3 in the sensitive but not resistant cells. These results are in accordance with a central modulatory role of Xiap as a caspase inhibitor and in Fas-mediated death signaling in cisplatin-resistance in ovarian cancer cells.

Recent studies have shown that cell adhesion is an important cell survival determinant and that the loss of cell–cell or cell–matrix contact, known as *anoikis* (Greek for homelessness), induces apoptosis (Frisch and Francis, 1994). While cancer cells are capable of invading surrounding tissue and implanting in distant site via blood stream or peritoneal dissemination, derangement of cell adhesion molecules has been reported in various cancer cells, including those of the ovary (Buczek-Thomas et al., 1998). Cisplatin-induced apoptosis is believed to be cell density-dependent (Takemura et al., 1991), although the precise mechanism of this phenomenon and the effect of cisplatin on cell adhesion are not fully understood. Focal adhesion kinase (FAK), a 125-kDa non-receptor protein tyrosine kinase involved in integrin-mediated cell adhesion, is a downstream effector of the integrin signaling pathway. Upon association with integrin, FAK is autophosphorylated and activated and apoptosis is suppressed. The anti-apoptotic action of FAK is mediated via its binding to other signal transduction molecules required for activation of the Ras-mitogen activated protein kinase (MAPK) cascade and of the PI3K/Akt pathway or cytoskeletal proteins in the formation of focal adhesion. It has been demonstrated that FAK suppresses anchorage-dependent apoptosis (Frisch et al., 1996) and that inhibition of FAK induces apoptosis (Hungerford et al., 1996) and reduces cell motility (Ilic et al., 1996). FAK is proteolytically cleaved during apoptosis induction and caspases have been suggested to be involved (Crouch et al., 1996).

Similarly, the possible role of FAK processing and its regulation in a cisplatin-sensitive human ovarian epithelial cancer cell line (OV2008) have been examined (Sasaki et al., 2002). Treatment of cancer cells with cisplatin results in caspase-3-mediated FAK cleavage, cell detachment from the growth surface and apoptosis. Addition of active caspase-3 to the whole cell lysate elicited a similar pattern of FAK cleavage. FAK fragments were detected exclusively in cells detached from the growth surfaces. Furthermore, inhibition of caspase-3 attenuated cisplatin-induced FAK cleavage and apoptosis. FAK processing is modulated by Xiap and may play an important role in cell detachment and termination of the integrin-mediated anti-apoptotic signal. These findings support the hypothesis that Xiap-mediated resistance of ovarian cancer cells to cisplatin may in part be due to Xiap

suppression of FAK processing and ultimately the maintenance of cell–matrix interaction (Fig. 2A and B, Sasaki et al., 2002).

4. Endogenous regulators of Xiap function

The caspase-inhibiting activity of Xiap is negatively regulated by at least three intracellular Xiap-interacting proteins: Xiap-associated factor 1 (XAF1), DIABLO/Smac and HtrA2/Omi (Liston et al., 2001; Srinivasula et al., 2000; Du et al., 2000; Verhagen et al., 2000; Suzuki et al., 2001). XAF1 is a nuclear protein that directly inhibits the anti-caspase activity of Xiap. XAF1 resides in the nucleus and can effect the re-localization of either endogenous or over-expressed XIAP protein from the cytoplasm to the nucleus. It functions as a negative regulator of the IAPs (Liston et al., 2001). Xiap suppresses caspase activation and cell death in vitro, and XAF1 antagonizes these activities. XAF1 is either not expressed, or present only in low levels in most cancer cell lines, suggesting that alterations in the balance of IAP and caspase activities is a common occurrence in the development of the transformed state. It has also been proposed that limited activation of caspases may be required for some normal cellular processes, such as T-cell activation, and may be initiated via tightly controlled expression of XAF1 (Liston et al., 2001). XAF1 does not require an activation signal, and appears to constitutively interact with Xiap and inhibit its function in healthy cells.

DIABLO/Smac is a mitochondrial protein that is released into the cytosol in response to apoptotic triggers. The protein is able to promote cytochrome c-mediated apoptosis by binding to and inhibiting Xiap (Du et al., 2000; Verhagen et al., 2000). Unlike XAF1, the functional domains of DIABLO/Smac have been established (Srinivasula et al., 2000; Du et al., 2000). Amino terminal sequences in DIABLO/Smac are required for Xiap inhibition. Indeed, mutation of the very first amino acid abolishes DIABLO/Smac function (Du et al., 2000). While BIR3 and BIR2 of Xiap are associated with caspase-9 and caspase-3 inhibition, respectively, four NH_3 terminal amino acids (Ala-Val-Pro-Ile) in DIABLO/Smac interact with the BIR3 domain of Xiap. Furthermore, wild-type DIABLO/Smac can act as a dimer to bind the BIR2 domain of Xiap. Over-expression of either DIABLO/Smac or XAF1 does not appear to induce apoptosis, but does sensitize cells to additional cell death triggers (Liston et al., 2001).

More recently, a serine protease, called HtrA2/Omi, has been reported to be released from the mitochondria and to inhibit Xiap function in a manner similar to that of DIABLO/Smac (Suzuki et al., 2001). Moreover, when overexpressed outside mitochondria, HtrA2/Omi induces atypical cell death, which is neither accompanied by a significant increase in caspase activity nor inhibited by caspase inhibitors, including Xiap (Suzuki et al., 2001). These interesting findings support the contention that in addition to its role as a caspase activator, HtrA2/Omi is also a caspase-independent cell death inducer, the action of which involves serine protease activation.

5. FLIP expression and resistance to TNFα- and trail-induced apoptosis

Tumor necrosis factor-α (TNFα) is both a death and survival factor in various cell lineages (Baker and Reddy, 1996), but the molecular and cellular basis for this phenomenon is not completely understood. The action of TNFα is mediated by its receptors, TNFR1 and TNFR2, and binding of TNFα to its receptors activates caspase-8 and caspase-3 (Boldin et al., 1996; Chinnaiyan et al., 1996; Medema et al., 1997; Yang et al., 1998) as well as induces IκB phosphorylation and degradation, and activates NFκB (Kruppa et al., 1992; Laegreid et al., 1994; Rothe et al., 1994; Berberich et al., 1994; Sarma et al., 1995).

It has been demonstrated that the inability of TNFα to induce apoptosis is due to the induction of survival factors, including IAPs (Erl et al., 1999; Stehlik et al., 1998; Xiao et al., 2001) and members of the Bcl-2 family (Grimm et al., 1996; Zong et al., 1999). Although Xiap is important in determining the apoptotic responsiveness of ovarian granulosa cells to TNFα (Xiao et al., 2001), it appears to play a minimal role, if any, in conferring resistance of the human ovarian cancer cell OV2008 to the cytotoxic action of the cytokine, as TNFα decreases Xiap content in the ovarian cancer cells. However, it is of interest to note that, in the presence of protein synthesis inhibitor CHX, TNFα induced Xiap cleavage in OV2008 cells, a process sensitive to the presence of the caspase inhibitors ZVAD and DEVD. These findings, together with the observations that cleavage of Xiap produces an N-terminal BIR-2 fragment with reduced ability to inhibit caspase-3 and suppress apoptosis (Deveraux et al., 1999), support the contention that the caspase-3-mediated processing of Xiap may in part be involved in the execution of apoptosis in ovarian cancer cells in response to TNFα.

Flice-like inhibitory protein (FLIP) is an intracellular anti-apoptotic protein, which modulates the cell surface receptor-mediated cell death process by inhibiting the activation of caspase-8 (Kreuz et al., 2001; Micheau et al., 2001). FLIP is a FADD-binding suppressor of apoptosis and is present as long ($FLIP_L$) and short ($FLIP_S$) isoforms. Both isoforms contain two death effector domains (DED), a structure resembling the N-terminal half of caspase-8 (Thome et al., 1997; Hu et al., 1997; Goltsev et al., 1997). Through DED–DED interaction. FLIP functions as dominant negative for FLICE, and blocks Fas-mediated apoptosis by preventing the activation of caspase-8 (Hu et al., 1997; Irmler et al., 1997; Srinivasula et al., 1999). Recent studies have demonstrated a role of FLIP and regulation of its expression by TNFα in a human ovarian cancer cell line (OV2008) in vitro. While TNFα alone was incapable of

inducing cell death, it remarkably increased the apoptotic cell number, caspase-8 and -3 cleavage in the presence of the protein synthesis inhibitor cycloheximide. TNFα also induced NFκB-mediated expression of $FLIP_S$, but not of $FLIP_L$. While down-regulation of $FLIP_S$ expression by $FLIP_S$ antisense cDNA facilitated TNF-induced cell death, over-expression of $FLIP_S$ sense attenuated TNF-induced apoptosis in the presence of cycloheximide. This study demonstrated that TNFα up-regulates $FLIP_S$ expression and this effect is mediated by the activation of NFκB. The induction of $FLIP_S$ expression by TNFα might contribute to the resistance of OV2008 cells to the pro-apoptotic action of the cytokine (Xiao, C.W., Li, Y., Reddy, S.A.G. and Tsang, B.K., unpublished observations).

It is of interest to note that the role of FLIP in conferring resistance to cell surface receptor-mediated apoptosis is not confined to the action of TNFα. The TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family and an apoptosis inducer in tumor but not normal cells via death receptors DR4 and DR5 (Suliman et al., 2001). The differences in sensitivity of various transformed and cancer cells to the pro-apoptotic action of TRAIL appear to be associated with the differences in $FLIP_L$ expression (Leverkus et al., 2000; Griffith et al., 1998). In addition, antigen receptor signaling is known to up-regulate $FLIP_L$ in primary B cells and to suppress the Fas- and TRAIL-receptor mediated apoptosis (Wang et al., 2000) and Fas-mediated apoptosis associated with the pathophysiology of rheumatoid arthritis is regulated at the level of caspase-8 through increased FLIP expression (Kobayashi et al., 2000). Moreover, c-$FLIP^{-/-}$ embryonic fibroblasts are highly sensitive to FasL- or TNF-induced apoptosis and show rapid induction of caspase activities, suggesting that c-FLIP mediates cytoprotection against death factor-induced apoptosis (Yeh et al., 2000). Whether FLIP has a role in conferring resistance of ovarian cancer cells to the cytotoxic action of TRAIL remains to be determined.

6. PI3K/Akt pathway in cancer cells

Phosphoinositide 3-kinase (PI3K) is a heterodimer composed of a p85-regulatory and a p110-catalytic subunit. In mammalian cells, three isoforms of p85 and p110 have been cloned, namely p85α, p85β, p85γ, p110α, p110β, and p100γ, and found to differ in their activation mechanisms by extracellular agonists, substrate specificity, and subcellular and tissue distribution. PI3K phosphorylates inositol lipids at the 3′ position of the inositol ring to generate the 3-phosphoinositides PtdIns-3-P, PtdIns-3,4-P2, and PtdIns-3,4,5-P3. Type III PI3Ks are responsible for the synthesis of PtdIns-3-P, which is constitutively present in all cells, and its levels do not dramatically change following stimulation. In contrast, PtdIns-3,4-P2 and PtdIns-3,4,5-P3, generated by types I and II enzymes, are normally undetectable in most cells. Their levels rapidly accumulate upon stimulation that activates downstream molecules including pleckstrin homology (PH) domain-containing proteins PDK1 and Akt, as well as PKC, JNK1, and p38 (Leevers et al., 1999) to mediate a variety of cellular responses including cell growth and transformation, differentiation, motility, insulin action, and cell survival (Fig. 3).

Numerous studies have shown that Akt is a major target of PI3K. Akt, also known as protein kinase B (PKB), is a subfamily of serine/threonine protein kinases (Cheng et al., 1992; Bellacosa et al., 1991; Jones et al., 1991a,b; Nakatani et al., 1999). Three members, Akt/AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ, have been identified in this family. While AKT1 is the true human homologue of v-akt/mouse Akt (98% identity at the amino acid level), AKT2 is a v-akt closely related kinase (Cheng et al., 1992). The three isoforms of Akt/PKB are highly homologous to v-akt. The overall homology of between these three isoforms is >85%. They share a very similar structure, which contains an N-terminal PH domain, a central kinase domain, and a serine/threonine-rich C-terminal region. The PH

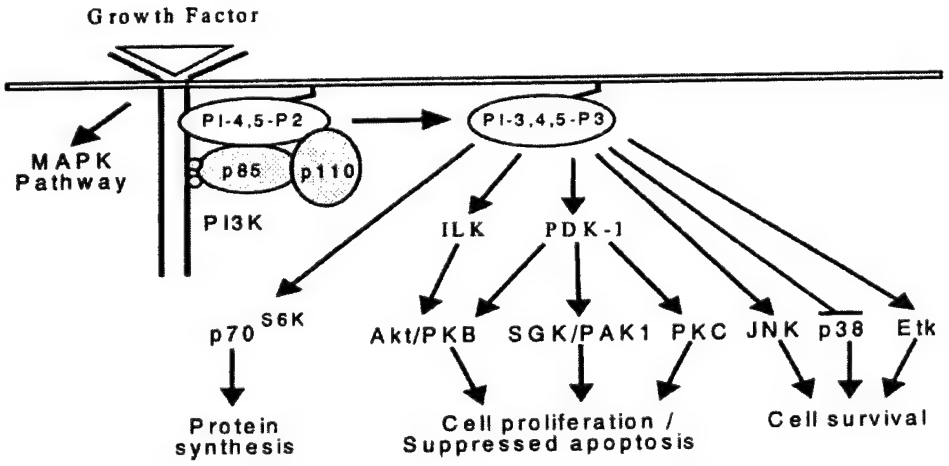
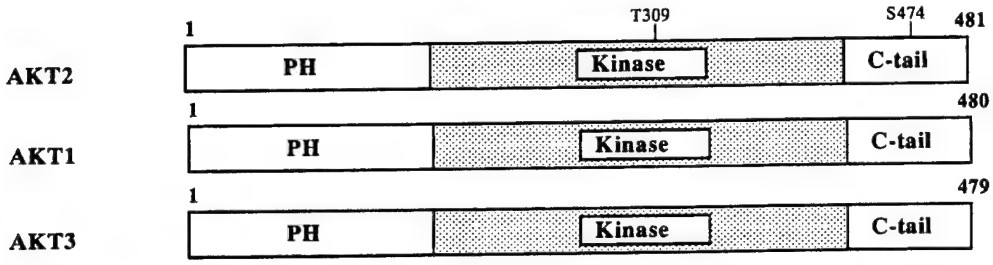


Fig. 3. The PI3K pathway and major downstream targets of PI3K.



AKT2 to AKT1	84%	95%	79%
AKT2 to AKT3	75%	90%	73%
AKT1 to AKT3	82%	91%	83%

Fig. 4. Diagrammatic representation of the structure of Akt family members. The percentages indicate the degree of homology between AKT1, AKT2 and AKT3 proteins in three different domains: PH, kinase, and regulatory (C-tail) regions.

domain and C-terminal region between these three isoforms are more diverse (homology 73–84%) as compared to the kinase domain (homology 90–95%), suggesting that PH and C-terminal regions may represent functional difference between A/Akt, AKT2 and AKT3 (Fig. 4).

It has been shown that Akt is activated by a variety of stimuli in a PI3K dependent manner and is essential for cell survival (Franke et al., 1995; Burgering et al., 1995; Meier et al., 1997; Liu et al., 1998). Activation of Akt by growth factors depends on the integrity of the PH domain, which binds to PI3K products PtdIns-3,4-P2 and PtdIns-3,4,5-P3, and on the phosphorylation of Thr^{308} (Thr^{309} in AKT2 and Thr^{305} in AKT3) in the activation loop and Ser^{473} (Ser^{474} in AKT2 and Ser^{472} in AKT3) in the C-terminal activation

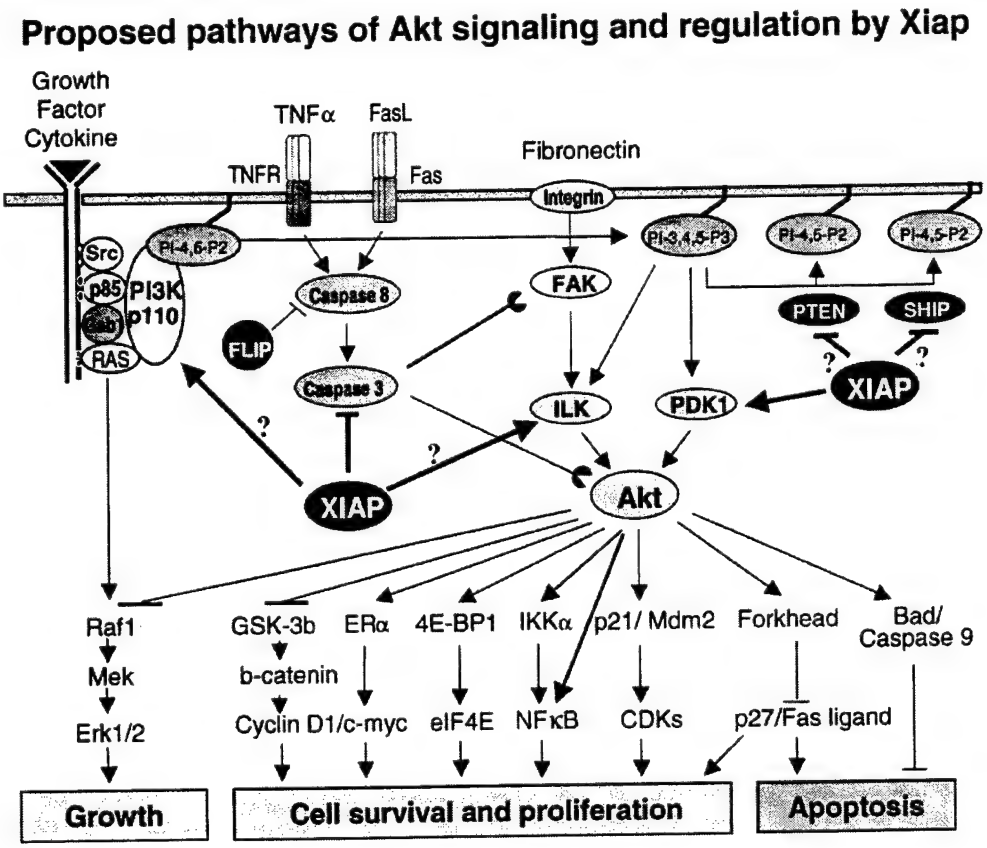


Fig. 5. Proposed pathways of cell signaling and regulation by Xiap. Activation of Akt is mediated by PI3K. In addition, activated Ras, Src and Gabi as well as Xiap have been shown to induce PI3K/Akt pathway. Ligation of Fas and TNF receptor (TNFR) leads to the activation of caspase-8 and caspase-3, and caspase-3-mediated cleavage of FAK and Akt.

domain by PDK1 (Fig. 5, Chan et al., 1999). The activity of Akt is negatively regulated by *PTEN*, a tumor suppressor gene that is mutated in a number of human malignancies including human endometroid ovarian carcinoma. *PTEN* encodes a dual-specificity protein and lipid phosphatase that reduces intracellular levels of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 in cells by converting them to PtdIns-4-P1 and PtdIns-4,5-P2 respectively, thereby inhibiting the PI3K/Akt signaling pathway (Li et al., 1998; Stambolic et al., 1998).

Although AKT1, AKT2, and AKT3 display high sequence homology and share similar upstream signal regulators and downstream targets, there are clear differences between the three isoforms in terms of biological and physiological function: (a) *AKT1* expression is relatively uniform in various normal organs whereas high levels of *AKT2* and *AKT3* mRNA are detected in skeletal muscle, heart, placenta and brain (Cheng et al., 1992; Nakatani et al., 1999; Bellacosa et al., 1993; Altomare et al., 1995); (b) overexpression of wild type AKT2, but not AKT1, transforms NIH3T3 cells (Cheng et al., 1997; Ahmed et al., 1993); (c) AKT2 and AKT3, but not AKT1, are amplified and/or upregulated in certain types of human cancer, such as carcinoma of ovary, breast, and pancreas (Cheng et al., 1992, 1996; DeFeo et al., 1995; Miwa et al., 1996; Ruggeri et al., 1998; Nakatani et al., 1999); (d) AKT1 and AKT3, but not AKT2, are activated by heat shock and H_2O_2 (Shaw et al., 1998); and (e) Akt2- and Akt1-deficient mice displayed different phenotypes. A recent study demonstrated that knockout mice deficient in Akt2 are impaired in the ability of insulin to lower blood glucose because of defects in the action of the hormone on skeletal muscle and liver. $Akt2^{-/-}$ mice are born without apparent defects, but develop peripheral insulin resistance and non-suppressible hepatic glucose production, resulting in hyperglycemia accompanied by inadequate compensatory hyperinsulinemia (Cho et al., 2001a), similar in some important features to type 2 diabetes in human. These phenotypic characteristics are not compensated by the presence of Akt1 and Akt3, reflecting differences of substrate specificity in insulin-responsive tissues. In contrast, *Akt1*-deficient mice did not display a diabetic phenotype (Chen et al., 2001; Cho et al., 2001b). The mice are viable but display impairment in organismal growth, i.e. are smaller when compared to wild type littermates. Such relatively subtle phenotypic change in $Akt1^{-/-}$ mice suggests that Akt2 and Akt3 may substitute to some extent for Akt1 (Chen et al., 2001). Nevertheless, the data indicate that there are non-redundant functions between the three isoforms of Akt in certain tissues and/or cell types.

7. PI3K and Akt in ovarian cancer chemoresistance

Alterations of PI3K in human maligriancies have been demonstrated including carcinomas of ovary, colon, breast and lung (Shayesteh et al., 1999; Yuan et al., 2000; Phillips et al., 1998; Moore et al., 1998; Shaw et al., 1997; Sun et al., 2001b). Among downstream targets of PI3K, only the Akt family has been implicated in malignant transformation (Cheng et al., 1997; Mende et al., 2001). We previously demonstrated alterations of AKT2 at DNA and mRNA levels in 15–20% of human ovarian cancer (Cheng et al., 1992; DeFeo et al., 1995) and recently documented frequent activation/overexpression of AKT2 (36%/46%) in 91 primary ovarian carcinomas by an in vitro kinase assay (Yuan et al., 2000). We have also shown that activation of Akt in human ovarian carcinoma resulted from overexpression and/or activation of PI3K and that inhibition of PI3K induced programmed cell death in ovarian cancer cell lines overexpressing AKT2 (Yuan et al., 2000; Sun et al., 2001a). The majority of cases with PI3K/Akt alterations are high grade and late stage tumors, implying that PI3K/Akt is associated with ovarian cancer progression rather than initiation. These studies suggest that PI3K/Akt pathway may play a pivotal role in ovarian carcinogenesis.

Recent studies indicated that expression of Xiap and HER-2/neu rendered tumor cells resistant to TNFα or chemotherapeutic agents by activation of PI3K/Akt pathway (Asselin et al., 2001; Zhou et al., 2000a). A report further demonstrated that ovarian cancer cells either overexpressing constitutively active Akt/AKT1 or containing *AKT2* gene amplification were far more resistant to paclitaxel than cancer cells expressing low AKT levels (Page et al., 2000). We have recently observed that cisplatin-sensitive ovarian cancer cells (A2780s and OV2008) transfected with constitutively active AKT2 became resistant to cisplatin, whereas overexpression of dominant negative AKT2 rendered cisplatin-resistant ovarian cancer cells (A2780cp and C13) susceptible to cisplatin-induced apoptosis (unpublished data). In addition, we previously reported inhibition of tumorigenicity of pancreatic cancer cell lines by antisense AKT2 (Cheng et al., 1996) and recently demonstrated that PI3K/AKT2 is a critical target for farnesyltransferase inhibitor (FTI)-induced apoptosis and constitutively active PI3K and AKT2 overcame FTI-277-induced programmed cell death (Jiang et al., 2000). Taken together, these data indicate that the PI3K/Akt pathway is a critical target for ovarian cancer intervention and that activation of this pathway is associated with chemoresistance in human ovarian carcinoma. Mechanisms for PI3K/Akt mediated chemoresistance include suppression of apoptosis, cell cycle progression, and angiogenesis.

7.1. Suppression of apoptosis

In numerous cell types, it has been shown that Akt induces survival and suppresses apoptotic death induced by a variety of stimuli. A major identified target of Akt is Bad that is phosphorylated by Akt at Ser-136 (Jiang et al., 2000; del Peso et al., 1997; Datta et al., 1997). It has also been shown that Akt activates PAK1 that in turn phosphorylates Bad at Ser-112 resulting in its release from $Bcl\text{-}_{xL}$ complex.

Furthermore, Akt phosphorylates Forkhead transcription factors and IκB kinase α (IKKα) leading to inhibition of Fas ligand transcription and activation of the NF-κB cascade (Datta et al., 1999). It has also been shown that Akt could target at the postmitochondrial level, as expression of constitutively active Akt failed to inhibit cytochrome c release induced by DNA damaging agents (Zhou et al., 2000b). The mechanism could include either enhancement of Xiap function or inhibition of Xaf-1, DIABLO/Smac or HtrA2 activity. Indeed, our recent data show that activation of Akt induces Xiap expression (see below).

7.2. Cell cycle progression

Akt phosphorylates glycogen synthase kinase (GSK)-3β and ERα resulting in upregulation of c-myc and cyclin D1 (Sun et al., 2001b; Datta et al., 1999). The cyclin-dependent kinase (CDK) inhibitor p27 is also down-regulated by Akt at both the transcription and posttranslation levels (Medema et al., 2000). Moreover, Akt phosphorylates and abrogates p21 function (Rossig et al., 2001). Recent studies also documented that Akt targets MDM2 to the nucleus, resulting in decreases of protein level and transcriptional activity of p53 (Mayo and Donner, 2001).

7.3. Angiogenesis

Accumulated evidence shows that Akt plays a central role in angiogenesis by mediating vascular endothelial growth factor (VEGF) signals and inducing transcription and translation of VEGF. It is well known that VEGF has various functions on endothelial cells, the most prominent of which is the induction of proliferation and differentiation by selectively binding to the Flk-1/KDR receptor and subsequent activation of PI3K/Akt pathway (Gerber et al., 1998). Moreover, constitutively active Akt can induce VEGF mRNA expression by stabilization (Zhong et al., 2000) and enhancing translation (Laughner et al., 2001) of HIF1α, which translocates to the nucleus, dimerizes with UIF1β and activates VEGF transcription.

8. Interactions between Xiap and PI3K pathway as possible mechanisms for chemoresistance

Although PI3K, Akt and Xiap are important cell survival factors in ovarian cancer cells (Li et al., 2001; Sasaki et al., 2000; Chan et al., 1999; Brunet et al., 2001), if and how they interact to confer resistance to chemotherapy is not known. Our laboratories have recently investigated the role of Xiap in the regulation of PI-3Kinase/Akt cell survival pathway in chemosensitive (A2780-s, OV2008 and OVCAR-3) and resistant (A280-cp) ovarian cancer cell lines and the nature of this interaction in cell death/survival signaling (Asselin et al., 2001). During a 24-h culture period, cisplatin decreased Xiap protein levels, activated caspase-3 and -9, and induced Akt cleavage and apoptosis in chemosensitive but not in resistant ovarian cancer cells (Li et al., 2001; Asselin et al., 2001). The Akt cleavage appeared to be mediated through caspase-3 activation, as human recombinant active caspase-3 was able to cleave Akt in vitro. The cisplatin-induced activation of caspase-9 and caspase-3 was blocked by Xiap over-expression (Asselin et al., 2001). Xiap down-regulation by antisense expression also induced Akt cleavage and apoptosis. Pretreatment ovarian cancer cells and their whole cell lysate with tetrapeptide inhibitors of caspase (e.g. DEVD) in vitro significantly decreased Akt cleavage induced by cisplatin and exogenous active caspase-3, respectively (Asselin et al., 2001). These results clearly demonstrate that Xiap plays an important role in the regulation of cisplatin sensitivity of ovarian cancer cells in part by modulating caspase-3-mediated cleavage of Akt, and thus, the integrity and function of the PI 3-kinase/Akt cell survival pathway.

We have also demonstrated that Xiap prevents apoptosis through up-regulation of PI3K/Akt cell survival signaling pathway and that the site of action of Xiap is in part upstream of Akt. Specifically, over-expression of Xiap by adenoviral sense Xiap cDNA infection increased phospho-Akt content (indicative of Akt activation), which was associated with a decrease in cisplatin-induced apoptosis (Asselin et al., 2001). However, in the presence of the PI3K inhibitor LY294002, Xiap over-expression failed to block cisplatin-induced apoptosis and to increase phospho-Akt content (Asselin et al., 2001), suggesting that the site(s) of action of Xiap on the PI3K/Akt pathway is at and/or proximal to PI3K. Whether Xiap up-regulates this pathway by turning on kinases (e.g. PI3K, integrin-linked kinase (ILK) and phosphoinositide-dependent kinase 1 (PDKI)) and/or down-regulating phosphatases (e.g. SH2-containing inositol polyphosphate-5-phosphatase (SHIP) and phosphatase and tensin homology (PTEN)) is not known (Fig. 5). Further, Akt has recently been shown to participate in the regulation of both p53-dependent and p53-independent p21 expression in ovarian cancer cells (Mitsuuchi et al., 2000) and that the induction of apoptosis by antisense Xiap expression is dependent on the p53 status of the cells. However, it is not known whether the regulation of the PI3K/Akt pathway by Xiap and its role to cisplatin-resistance is p53 dependent. Nevertheless, these findings offer a new mechanism by which Xiap may regulate apoptosis and confer cisplatin-resistance in ovarian cancer cells.

In addition, we have recently observed that Akt induces Xiap expression at the protein level in HeLa and ovarian cancer cells transfected with constitutive active Akt in a tetracycline-inducible system. Although the mechanism(s) of this regulation is not known, it may possibly include (a) increased Xiap promoter activity via activation of the NFκB pathway, (b) phosphorylation of $p70^{S6K}$ and 4E-BP1, leading to the induction of Xiap translation and (c) inhibition of Xiap degradation. Nevertheless, Akt-induced Xiap expression supports the notion that Akt exerts its anti-apoptotic

effect at the post-mitochondrial level (Zhou et al., 2000b), and could be a major determinant for activation of PI3K/Akt pathway-associated chemoresistance in human ovarian cancer. The notion that Akt may play a role in the regulation of Xiap expression is supported by our recent observation that the follicle stimulating hormone-induced, NFκB-mediated Xiap expression in rat ovarian granulosa cells is sensitive to the PI3K inhibitor LY294002 in vitro (Wang et al., 2001).

9. Xiap and PI3K/Akt pathway as potential therapeutic targets

As elaborated earlier, the ability of a therapeutic agent to induce apoptosis is a critical factor in the success of ovarian cancer chemotherapy. Although cisplatin and taxol derivatives are first-line chemotherapeutic agents for ovarian cancer, chemoresistance remains a major therapeutic hurdle. The prospect of gene manipulation as an alternative therapeutic approach for ovarian cancer, particularly for the chemoresistant disease, has generated considerable excitement. Adenovirus has been the most promising vehicle for gene replacement, but the use of non-DNA-based viruses is also being explored. Recent novel advances in this therapeutic approach include refinement of vector targeting and the use of site-specific promoters and conditionally replicative adenoviral vectors (Collinet et al., 2000). Of the 380 gene therapy clinical trials (3173 patients) currently in progress or to be initiated, 16 trials are related to ovarian cancer and involve mutation compensation by replacement of an altered tumor suppressor gene, molecular therapy by suicide gene or multi-drug resistance gene transfer, and oncogene inhibition (Collinet et al., 2000). Although several clinical trials have documented the relative safety of gene therapy in ovarian cancer patients, few significant clinical responses have been effected. Targeting Xiap or its function has not been explored.

We have recently shown that down-regulation of Xiap with an adenovirus vector containing a Xiap antisense cDNA sensitizes cisplatin-resistant ovarian cancer cell lines to the pro-apoptotic action of cisplatin (Sasaki et al., 2000). As over 75% of ovarian cancers carry a mutated and non-functional *p53* gene and the induction of apoptosis by Xiap antisense expression is p53-dependent (involves caspase-dependent p53 accumulation), down-regulation of Xiap alone would have limited value as a therapeutic strategy. However, the observation that Xiap down-regulation enhances the apoptotic response induced by adenoviral WT-p53 sense expression raises the interesting possibility that down-regulation of Xiap expression or function could serve as an important adjuvant for *p53* gene therapy (Sasaki et al., 2000). These results provide the necessary proof of principle for Xiap as an important etiological factor in cisplatin resistance in ovarian cancer and demonstrate Xiap may be a target for gene or molecular therapy. It should be noted, however, that antisense expression as an effective treatment modality in vivo is often problematic and unreliable. However, the expression of a dominant negative, which inhibits Xiap function may prove to be a more promising strategy.

Xiap is negatively regulated by at least three endogenous proteins: XAF1, DIABLO/Smac and HtrA2/Omi (Liston et al., 2001; Srinivasula et al., 2000; Suzuki et al., 2001). In vitro studies have shown that the prevention by Xiap of apoptosis in fibroblasts following serum withdrawal was dose-dependently antagonized by the *xaf1* gene, introduced using an adenovirus vector (Liston et al., 2001). It is possible, therefore, that over-expression of the *xaf1* gene may be a more effective mechanism of overcoming Xiap-induced chemoresistance. Introduction of the wild-type full-length XAF1 protein by adenoviral *xaf1* gene delivery may be an option. However, elucidation of the functional components of the protein may allow the design of small peptides containing the XAF1 domains crucial for Xiap sequestration. These small peptides would theoretically possess the same ability to sequester Xiap as the wild-type XAF1 protein, but could be readily taken up by the cells. The recent identification of the functional motif of DIABLO/Smac raises the possibility that small peptides which inhibit either or both of the BIR2 and BIR3 domains of Xiap, may potentially be promising candidates for future anti-cancer agents. In this context, it is of interest to note that a short 7-residue peptide derived from the DIABLO/Smac NH_3-terminal, which can promote the activation of procaspase-3, has already been reported (Wu et al., 2000).

Furthermore, current evidence suggests that overexpression of XAF1 or DIABLO/Smac does not, by itself, induce apoptosis. These factors, however, can sensitize cells to the actions of apoptotic stimuli. In this regard, using these peptides as models for novel anti-cancer agents may prove useful in that they are not expected to cause unwanted cell death in the absence of a chemotherapeutic agent. The therapeutic potential could possibly be realized with heightened sensitivity to chemotherapy following targeting of the exogenous peptide with cell-specific promoters. The search for ovarian cell-specific promoters for ovarian cancer is on-going and the recent demonstration that OSP-1 promoters can successfully drive the expression of a *lacZ* gene in an ovarian-specific manner is particularly noteworthy (Garson et al., 2001; Selvakumaran et al., 2001).

Similar to DIABLO/Smac and XAF1, Omi/HtrA2 has been identified as a direct Xiap binding protein (Suzuki et al., 2001). Whether overexpression of all or part of the HtrA2 protein, either by genetic manipulation or by introduction of a small peptide containing the functional Xiap inhibition domain, can serve as a useful adjuvant to traditional chemotherapy in chemoresistant ovarian cancer remains to be determined. In addition, the importance of the serine protease-containing domain of HtrA2 in caspase-independent cell death induction and as a potential candidate for a therapeutic agent for ovarian cancer remains to be explored.

While it is known that Xiap may act through caspase inhibition and/or through the PI3K/Akt pathway, the relative contribution of each of these pathways to chemoresistance is not known. As discussed in the previous section, PI3K/Akt pathway is essential for cell survival, cell cycle progression and angiogenesis. Amplification/overexpression/activation of PIK3CA (p110α) enzymatic subunit of PI3K and Akt as well as somatic mutation of gene encoding p85α regulatory subunit of PI3K are frequently detected in human ovarian cancer (Shayesteh et al., 1999; Cheng et al., 1992; Yuan et al., 2000; Sun et al., 2001a; Philp et al., 2001). Inhibition of PI3K and/or Akt induces programmed cell death in ovarian cancer cells (Yuan et al., 2000). Expression constitutively active AKT2 results in ovarian cancer cells resistant to cisplatin, taxol, and FTIs-induced apoptosis, whereas dominant negative Akt sensitizes the cells to chemotherapeutic drugs (Herod et al., 1996; Datta et al., 1997). Thus, PI3K/Akt pathway is a critical target for ovarian cancer intervention and inhibition of PI3K and/or Akt could overcome a subset of chemoresistant ovarian cancers. Biological and pharmacological approaches have been tested to inhibit PI3K/Akt pathway.

Biological approaches include antisense, dominant-negative, antibody of PI3K and Akt as well as peptides to mimic and compete pleckstrin-homology (PH) domain of Akt binding to PI3K products, PtdIns-3,4-P2 and PtdIns-3,4,5-P3. We have previously demonstrated that the introduction of antisense AKT2 into several AKT2-overexpressing cancer cell lines abrogated endogenous AKT2 expression and diminished their invasiveness and tumour formation in nude mice (Cheng et al., 1996). Antisense oligonucleotides of Akt can inhibit Akt pathway and induce apoptosis in different cell lines (Liu et al., 2001) and cell growth and survival can also be inhibited by the expression of dominant negative (DN) forms of PI3K and Akt (Sato et al., 2000; Orik et al., 2001). Our recent data show that expression of DN-Akt in NIH3T3 cells remarkably reduces v-H-ras-induced colony formation and tumor formation (unpublished data). Moreover, consistent with the tumor-inhibitory effects of DN-PI3K and DN-Akt is the demonstration of the inhibition of Ras and BCR/ABL malignant transformation with p85ΔiSH2 and DN-Akt, respectively (Rodriguez-Viciana et al., 1997; Skorski et al., 1997). Microinjection of AKT2 antibody into myoblasts can specifically block their function, i.e. induction of myotube (Vandromme et al., 2001). Further studies are required to investigate the effects of antibodies of PI3K and Akt on human cancer cell growth.

The importance of PI3K and Akt in cell survival, growth, cell transformation and human malignancy has prompted the search for specific and safe pharmacological inhibitors for PI3K and Akt. Although wortmanin and LY294002 efficiently abrogate PI3K activity and have been widely used in the cell culture system (Dong et al., 1999; Mills et al., 2001), they have not been applied for clinical trails due to either toxicity (LY294002) or a short of half-life (wortmanin). To date, only one Akt inhibitor is commercially available (Calbiochem). However, there are not enough data to draw the conclusion now. We have recently demonstrated that FTI-277, originally designed to block Ras oncoprotein, inhibits PI3K/Akt pathway and induces apoptosis in a number of human cancer cell lines including ovarian carcinoma (Jiang et al., 2000). ETIs are highly effective at inhibiting tumor growth without toxicity to normal cells. However, the mechanism by which they inhibit tumor growth is not well understood (Cox and Der, 1997; Sebti and Hamilton, 1997; Lebowitz and Prendergast, 1998). FTIs are unable to induce apoptosis in Raf transformed NIH3T3 cells even though MAPK pathway is inhibited by FTIs (Cox and Der, 1997; Sebti and Hamilton, 1997), indicating that ETIs may target other cell survival pathway(s) regulated by Ras or other farnesylated proteins. Interestingly, our data showed that FTI-277 induces apoptosis only in AKT2-overexpressing human cancer cell lines. Furthermore, overexpression of AKT2, but not oncogenic H-Ras, sensitizes NIH3T3 cells to FTI-277; and a high serum level prevents FTI-277-induced apoptosis in H-*Ras*-but not *AKT2*-transformed NIH3T3 cells (Jiang et al., 2000). These data suggest that FTIs specifically target the PI3K/Akt pathway to inhibit tumor cell growth and may be candidate agents for reversing resistance of human ovarian cancer to cisplatin and taxol.

10. Conclusions and future directions

Despite numerous attempts made to improve the therapeutic outcome for ovarian cancer in the past decades, chemoresistance remains a key concern for successful management of this gynecologic malignancy. The advent of molecular and cellular techniques and their applications in research in the regulatory mechanisms of tumor cell fate (i.e. proliferation, differentiation, and apoptosis) has facilitated major advances in the current understanding of the pathobiology of chemoresistance. The recent demonstration that the cell survival intermediates Xiap, PI3K and Akt as key determinants of chemosensitivity in ovarian cancer cells has brought new excitement on their potential role as therapeutic targets and the emergence of new strategies for the treatment of chemoresistant ovarian cancer. It is essential that future work in this area is aimed at developing pharmacological reagents as well as genetic and biochemical approaches that not only identify novel roles for Xiap and Akt but also verify the physiological functions previously ascribed to these intermediates. The generation of a potent and specific Xiap and Akt inhibitors would certainly revolutionize the study of the processes mediated by Xiap and Akt in the same way inhibitors of MAP kinase kinase 1 activation (e.g. PD98059, PD184352, U0126) have on our understanding of processes regulated by the classical MAP kinase pathway. More importantly, such drugs or in combination with conventional chemotherapeutic agents would reasonably improve the outcome of ovarian cancer. Yet, there is still much to be learned about how Akt activity is regulated by Xiap. The mechanism by which

Akt promotes cell survival, especially via postmitochondrial molecules regulated by Akt, is still obscure. Another important question that has not been addressed is whether FTIs, which have been shown to induce apoptosis by inhibition of PI3K/Akt pathway (Jiang et al., 2000), target Xiap and overcome chemoresistance in a subset of ovarian cancer. In addition, while Xiap down-regulation enhances the apoptotic response of chemoresistant ovarian cancer cells induced by adenoviral wild type p53 sense expression in vitro, whether down-regulation of Xiap expression or function could indeed serve as an important adjuvant for *p53* gene therapy, remains to be tested. The inhibition of Xiap function in target cells via expression or delivery of Xiap dominant negatives (e.g. XAF1, DIABLO/Smac and HtrA2/Omi), may prove to be a promising strategy. The realization of these therapeutic potentials will rest upon the success in cell-specific gene targeting and delivery of the candidate "molecule." The outcome of these developments will have profound effects on the management of patients with drug-resistant ovarian cancer.

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AKT2 Inhibition of Cisplatin-induced JNK/p38 and Bax Activation by Phosphorylation of ASK1

IMPLICATION OF AKT2 IN CHEMORESISTANCE*

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Cisplatin and its analogues have been widely used for treatment of human cancer. However, most patients eventually develop resistance to treatment through a mechanism that remains obscure. Previously, we found that AKT2 is frequently overexpressed and/or activated in human ovarian and breast cancers. Here we demonstrate that constitutively active AKT2 renders cisplatin-sensitive A2780S ovarian cancer cells resistant to cisplatin, whereas phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 sensitizes A2780S and cisplatin-resistant A2780CP cells to cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway. AKT2 interacts with and phosphorylates ASK1 at Ser-83 resulting in inhibition of its kinase activity. Accordingly, activated AKT2 blocked signaling downstream of ASK1, including activation of JNK and p38 and the conversion of Bax to its active conformation. Expression of nonphosphorylatable ASK1-S83A overrode the AKT2-inhibited JNK/p38 activity and Bax conformational changes, whereas phosphomimic ASK1-S83D inhibited the effects of cisplatin on JNK/p38 and Bax. Cisplatin-induced Bax conformation change was inhibited by inhibitors or dominant negative forms of JNK and p38. In conclusion, our data indicate that AKT2 inhibits cisplatin-induced JNK/p38 and Bax activation through phosphorylation of ASK1 and thus, plays an important role in chemoresistance. Further, regulation of the ASK1/JNK/p38/Bax pathway by AKT2 provides a new mechanism contributing to its antiapoptotic effects.

Although cisplatin and its analogues, the DNA cross-linking agents, are first-line chemotherapeutic agents for the treatment of human ovarian and breast cancers, chemoresistance remains a major hurdle to successful therapy (1, 2). Several molecules have been implicated in cisplatin resistance, including decreased cellular detoxication (3, 4), increased DNA repair (5), and mutations of *p53* tumor suppressor gene (6, 7). However, the mechanisms involved in cisplatin resistance are still poorly understood. A growing body of evidence indicates that defects in the intra- and extracellular survival/apoptotic pathways are an important cause of resistance to cytotoxic agents.

Phosphatidylinositol 3-kinase (PI3K)[1]/Akt is a major cell survival pathway that has been extensively studied recently (8). PI3K is a heterodimer composed of a p85 regulatory and a p110 catalytic subunit and converts the plasma membrane lipid phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. Pleckstrin homology domain-containing proteins, including Akt, accumulate at sites of PI3K activation by directly binding to phosphatidylinositol 3,4,-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. Akt (also known as PKB) represents a subfamily of serine/threonine kinases. Three member of this family, including AKT1, AKT2, and AKT3, have been identified so far. Akt is activated in a PI3K-dependent manner by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress (9–12). Downstream targets of Akt contain the consensus phosphorylation sequence R*X*R*XX*(S/T)(F/L) (13). Several targets of Akt that have been identified have roles in the regulation of apoptosis, such as the proapoptotic proteins BAD and caspase-9 and transcription factor FKHRL1. Phosphorylation by Akt blocks BAD binding to Bcl-x_L, inhibits caspase-9 protease activity, and blocks FKHRL1 function, reducing Fas ligand transcription (14–16).

Among Akt family members, AKT2 has been shown to be predominantly involved in human malignancies including ovarian cancer. We have demonstrated previously amplification of the AKT2 in a number of human ovarian cancer cell lines and recently detected frequently elevated protein and kinase levels of AKT2 in about a half of primary ovarian carcinoma examined (17, 18). Moreover, ectopic expression of wild type of AKT2 but not Akt1 in NIH 3T3 cells resulted in malignant transformation (19). Inhibition of PI3K/AKT2 by farnesyltransferase inhibitor-277 induced apoptosis in ovarian cancer cells that overexpress AKT2 (20). We have also shown that TNFα and extracellular stresses, including UV irradiation, heat shock, and hyperosmolarity, induce AKT2 kinase and that

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[1] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun NH_2-terminal kinase; PARP, poly(ADP-ribose) polymerase; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; TNFα, tumor necrosis factor α; GST, glutathione *S*-transferase; HEK, human embryonic kidney; MKK, mitogen-activated protein kinase kinase.

activated AKT2 inhibits JNK/p38 activity to protect cells from TNFα and cellular stress-induced apoptosis (21).

JNK and p38 are predominantly activated through environmental stresses, including osmotic shock, UV radiation, heat shock, oxidative stress, protein synthesis inhibitors, stimulation of Fas, and inflammatory cytokines such as TNFα and interleukin-1. Stimulation of JNK/p38 activity has also been shown to be critical for cisplatin-induced apoptosis in some cancer cells (22, 23). Specific inhibition of JNK or p38, through small molecule inhibitors, dominant negative JNK/p38 mutants, or knock-out of JNK expression, suppresses various types of stress-induced apoptosis (24). Although it has been shown that JNK phosphorylates and inhibits antiapoptotic protein Bcl-2 (25), the mechanism of JNK/p38 induction of apoptosis is still not well understood.

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase kinase kinase family that activates both the SEK1-JNK and MKK3/MKK6-p38 signaling cascades (26–28). ASK1 is a general mediator of cell death in responds to a variety of stimuli, including oxidative stress (29, 30) and chemotherapeutic drugs such as cisplatin and paclitaxel (22, 23). Ectopic expression of ASK1 induced apoptosis in various cell types (26, 28). Furthermore, disruption of the *ASK1* gene in mice causes a remarkable reduction in sensitivity to stress-induced cell death, such as that promoted by TNFα or oxidative stress (33). These data indicate that ASK1 plays a key proapoptotic function through promoting the sustained activation of JNK/p38 mitogen-activated protein kinases.

In the present study, we show that AKT2 activity promotes resistance to cisplatin-induced apoptosis in A2780S ovarian cancer cells through the inhibition of the ASK1/JNK/p38 pathway. In A2780S cells, we show that AKT2 complexes with and phosphorylates ASK1 at Ser-83 within a consensus Akt phosphorylation site on this molecule. This results in inhibition of ASK1 activity and the blocking of JNK and p38 activation. We also show that these latter activities are required for cisplatin-induced apoptosis in A2780S cells. Furthermore, in response to cisplatin, we observe that ASK1 and JNK/p38 promote Bax conformational change. Collectively, these studies indicate that AKT2 may be an important mediator of chemoresistance through its regulatory effects on the ASK1/JNK/p38/Bax pathway.

EXPERIMENTAL PROCEDURES

Reagents—Cisplatin, LY294002, and anti-Bax (6A7) were obtained from Sigma. DMEM and fetal bovine serum were purchased from Invitrogen. Anti-phospho-Akt (Ser-473), anti-cleaved PARP, anti-phospho-JNK (p54/44), anti-phospho-extracellular signal-regulated kinase 1/2(44/42), anti-phospho-p38, anti-phospho-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2, and anti-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 antibodies were obtained from Cell Signaling (Beverly, MA). GST-c-Jun and GST-ATF6 were also purchased from Cell Signaling. Anti-AKT2, anti-Bax, and anti-ASK1 were obtained from Santa Cruz Biotechnology. JNK inhibitor II and p38 inhibitor SB203580 were from Calbiochem.

Cell Culture and Cisplatin Treatment—The human epithelial cancer cell lines, A2780S and A2780CP, kindly provided by Benjamin K. Tsang at The Ottawa Hospital, and human embryonic kidney (HEK) 293 cells were cultured at 37 °C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum. The cells were seeded in 60-mm Petri dishes at a density of 0.5×10^6 cells per dish. After 24 h, cells were treated with cisplatin (20 μM) for the appropriate time as noted in the figure legends.

Expression Constructs—The cytomegalovirus-based expression constructs encoding wild type HA-AKT2 and constitutively active HA-Myr-AKT2 have been described previously (31). The $pcDNA_3$-HA-ASK1 construct was kindly provided by Hidenori Ihijo at Tokyo Medical and Dental University. HA-ASK1-S83A and ASK1-S83D, as well as dominant negative AKT2 with triple mutations (T309A, E299K, and S474A), were created using the QuikChange site-directed mutagenesis kit (Stratagene). JNK and p38 plasmids were obtained from Roger Davis at the University of Massachusetts.

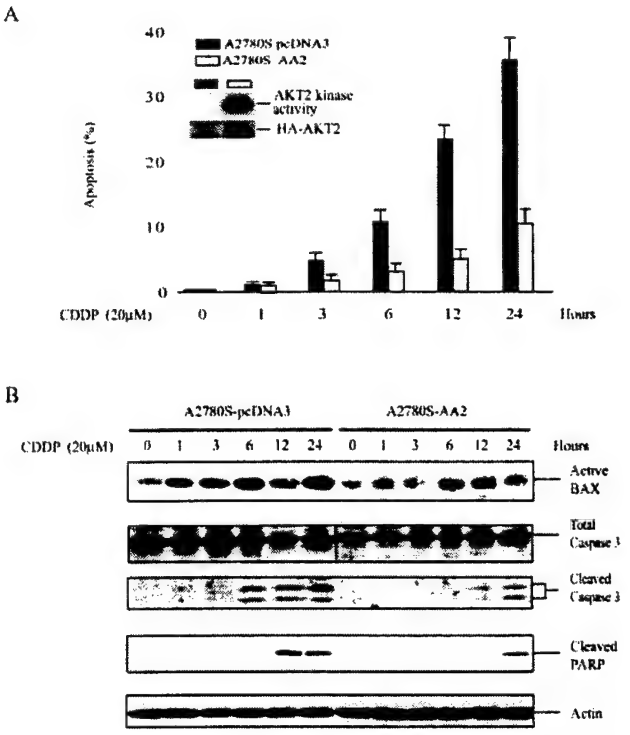

FIG. 1. **Activation of AKT2 renders cells resistant to cisplatin and inhibits cisplatin-induced Bax conformational change and caspase-3 cleavage.** *A*, cisplatin-sensitive A2780S cells were stably transfected with constitutively active AKT2 (*A2780S-AA2*) or pcDNA3 (*A2780S-pcDNA3*). Expression and kinase activity of transfected AKT2 were examined by Western blot and *in vitro* kinase assays (*inset*). The cells were treated with cisplatin (*CDDP*; 20 μM) for indicated time and analyzed by Tunel assay. Apoptotic cells were quantified in triple experiments. *B*, Western blot analysis. The cells were treated with cisplatin and lysed. A portion of lysate was immunoprecipitated with anti-active Bax (6A7) and detected with anti-total Bax antibody (*top panel*). The rest of the lysates were immunoblotted and probed with anti-caspase-3 (*second and third panels*), anti-PARP (*fourth panel*), and anti-actin (*bottom panel*) antibodies.

Immunoprecipitation and Immunoblotting—Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM NaPPi, 1 mM sodium vanadate, and 25 mM β-glycerolphosphate. Lysates were centrifuged at 12,000 $\times$ *g* for 15 min at 4 °C prior to immunoprecipitation or Western blot. Aliquots of the cell lysates were analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1)-agarose beads at 4 °C for 20 min. Following the removal of the beads by centrifugation, lysates were incubated with appropriate antibodies in the presence of 25 μl of protein A-protein G (2:1)-agarose beads for at least 2 h at 4 °C. The beads were washed with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and 0.5% Triton X-10; twice with phosphate-buffered saline; and once with buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol, all supplemented with 20 mM β-glycerolphosphate and 0.1 mM sodium vanadate. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates or total cell lysates with the appropriate antibodies as noted in the figure legends. Detection of antigen-bound antibody was carried out with the ECL Western blotting analysis system (Amersham Biosciences).

In Vitro Kinase Assay—Protein kinase assays were performed as described previously (21). Briefly, reactions were carried out in the presence of 10 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences) and 3 μM cold ATP in 30 μl of buffer containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. 2 μg of myelin basic protein was used as the exogenous substrate. After incubation at room temperature for 30 min the reaction was stopped by adding protein loading buffer, and proteins were separated on SDS-PAGE gels. Each

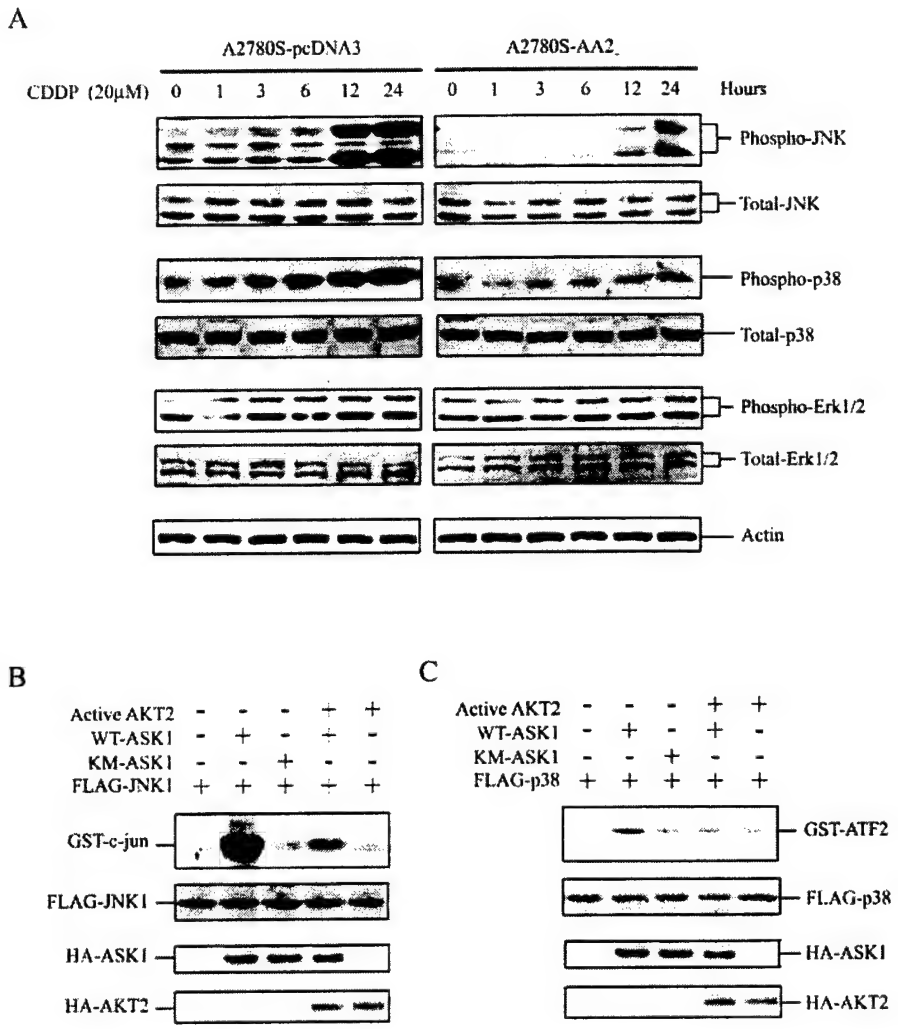

FIG. 2. **AKT2 inhibits JNK and p38 activation induced by cisplatin and ASK1.** *A*, immunoblotting analysis. Following treatment with cisplatin at indicated times, the cells were lysed and immunoblotted. The blots were detected with indicated antibodies. *B* and *C*, *in vitro* kinase assay. HEK293 cells were transfected with the indicated expression plasmids. After 36 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody. The FLAG-JNK and FLAG-p38 immunoprecipitates were subjected to *in vitro* kinase using GST-c-Jun (*B*) and GST-ATF2 (*C*) as substrate, respectively (*top panel*). Expression of the transfected plasmids was shown in the *second*, *third*, and *fourth panels*.

experiment was repeated three times, and the relative amounts of incorporated radioactivity were determined by autoradiography and quantified with a PhosphorImager (Molecular Dynamics).

In Vivo [^{32}P]P_i Labeling—HEK293 cells were co-transfected with active AKT2 and HA-tagged ASK1 or pcDNA3 and labeled with [^{32}P]P_i (0.5 mCi/ml) in phosphate- and serum-free DMEM medium for 4 h. Cell lysates were subjected to immunoprecipitation with anti-HA antibody. The immunoprecipitates were separated by 7.5% SDS-PAGE and transferred to membranes. Phosphorylated ASK1 band was visualized by autoradiography. The expression of transfected ASK1 was detected with anti-HA antibody.

Luciferase Reporter Assay—Cells were seeded in 6-well plate and transfected with c-Jun or ATF6 reporter plasmid (pGl-GAL4), pSV2-β-gal, and different forms (wild type, constitutively active, or dominant negative) of HA-AKT2 together with or without different forms of ASK1 or vector alone. After 36 h of the transfection, luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix, respectively). Each experiment was repeated three times.

Tunel Assay—Cells were seeded into 60-mm dishes and grown in DMEM supplemented with 10% fetal bovine serum for 24 h and treated with 20 μM cisplatin for different times. Apoptosis was determined by Tunel assay using an *in situ* cell death detection kit (Roche Applied Science). These experiments were performed in triplicate.

RESULTS

Activation of AKT2 Renders Cisplatin-sensitive Cells Resistant to Cisplatin and Inhibits Cisplatin-induced Bax Conformational Change—We have shown previously (18, 34) frequent activation of AKT2 kinase in human ovarian and breast cancers. To examine whether activation of AKT2 contributes to chemoresistance in cancer cells, cisplatin-sensitive A2780S cells were stably transfected with constitutively active AKT2 (A2780S-AA2) or pcDNA3 vector alone. Expression and kinase activity of transfected constitutively active AKT2 were confirmed by Western blot and *in vitro* kinase analysis (Fig. 1A, *inset*). Following treatment with cisplatin (20 μM) for 0, 1, 3, 6, 12, and 24 h, programmed cell death in A2780S-pcDNA3 and A2780S-AA2 (active AKT2) cells were examined by Tunel assay. The number of apoptotic cells was quantified by counting three different microscopic fields. Three h after treatment, A2780S-pcDNA3 cells begun to undergo apoptosis. By 24 h of treatment, 35% of the cells were apoptotic, which is a similar response reported in the literature for parental A2780S cells (35). However, we observed a distinctly lower percentage of apoptotic cells at the time points 3, 6, 12, and 24 h in A2780S-AA2 cells (Fig. 1A), indicating that activation of AKT2 renders cisplatin-sensitive A2780S cells resistant to cisplatin.

It has been shown that Bax is required for cisplatin-induced apoptosis, *i.e.* cisplatin activates Bax by inducing its N-terminal conformation change and then targeting it to mitochondria resulting in cytochrome *c* release and activation of apoptotic pathway (36, 37). Thus, we next examined the effects of AKT2 activation on induction of Bax conformational changes by cisplatin. After treatment with cisplatin, A2780S-pcDNA3 and A2780S-AA2 cells were lysed and immunoprecipitated with anti-active Bax (6A7) antibody. The immunoprecipitates were subjected to Western blot analysis with total anti-Bax anti-

body. As shown in Fig. 1*B*, cisplatin promotes alteration of Bax conformation after 3 h of treatment in A2780S-pcDNA3 cells but not in A2780S-AA2 cells. Accordingly, cleavage of caspase 3 and its substrate, PARP, was also inhibited by expression of constitutively active AKT2 as compared with pcDNA3-transfected A2780S cells (Fig. 1*B*).

AKT2 Inhibits Cisplatin- and ASK1-induced JNK and p38 Activation—It has been documented that stress kinases, JNK and p38, are activated by cisplatin, and their activations are required for cisplatin-induced programmed cell death (22, 23, 38). To examine whether the effect of cisplatin on JNK and p38 is abrogated by the activation of AKT2, A2780S-pcDNA3 and A2780S-AA2 cells were treated with cisplatin at different times. As expected, JNK and p38 were activated by cisplatin in A2780S-pcDNA3 cells, and the activation of p38 took place before that of JNK. However, the activation of JNK and p38 was reduced dramatically in A2780S cells transfected with a constitutively active AKT2. No significant difference in the phosphorylation levels of extracellular signal-regulated kinase was observed between these two cell lines (Fig. 2*A*).

To explore the mechanism of AKT2 inhibition of the JNK and p38, we probed for direct interaction of these proteins by coimmunoprecipitation. We were not, however, able to demonstrate any interaction between AKT2 and JNK or p38 (data not shown). As ASK1 is known to activate JNK/p38 and be induced by cisplatin (32), and its overexpression is sufficient to induce apoptosis (26, 28), we next examined whether AKT2 restrains JNK and p38 activity through inhibition of ASK1. HEK293 cells were transfected with FLAG-JNK1 or FLAG-p38 and wild type or kinase-dead ASK1 (KM-ASK1), with or without constitutively active AKT2. After 36 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody. FLAG-JNK1 and FLAG-p38 immunoprecipitates were subjected to *in vitro* kinase assays using GST-c-Jun and GST-ATF2 as substrates, respectively. Repeated experiments revealed that kinase activities of JNK1 and p38 were significantly induced by expression of wild type but not kinase-dead ASK1 and that the activation of JNK and p38 was attenuated by ectopic expression of constitutively active AKT2 (Fig. 2, *B* and *C*). These data indicate that AKT2 may negatively regulate ASK1, causing inhibition of cisplatin-induced JNK/p38 activation and apoptosis.

AKT2 Interacts with, Phosphorylates, and Inhibits ASK1—To examine whether ASK1 is a direct target of AKT2, co-immunoprecipitation was carried out with anti-AKT2 antibody and detected with anti-ASK1 antibody, and vice versa. As shown in Fig. 3, *A* and *B*, interaction between ASK1 and AKT2 was readily detected, and this interaction was enhanced by cisplatin treatment. Sequence analysis revealed that an AKT2 phosphorylation consensus site resides in ASK1 at residue Ser-83, which is conserved between human and mouse. To determine whether AKT2 phosphorylates ASK1, *in vitro* AKT2 kinase assays were performed using immunoprecipitated HA-ASK1 (wild type ASK1 or ASK1S83A) as substrates (Fig. 3*C*). In addition, *in vivo* [^{32}P] labeling and immunoblotting analyses with anti-phospho-Ser/Thr Akt substrate antibody were curried out in HEK293 cells transfected with ASK1 and constitutively active or wild type AKT2 (Fig. 3*D*). Both *in vitro* kinase and *in vivo* labeling experiments, as well as Western blot analysis, showed that wild type and constitutively active AKT2 phosphorylate ASK1 at Ser-83 with the lower phosphorylation level by wild type AKT2 (Fig. 3, *C* and *D*).

We next determined whether cisplatin-induced ASK1 activation is inhibited by AKT2 and, if it is, whether this inhibition depends upon AKT2 phosphorylation of ASK1 at Ser-83. Mutagenesis was used to create a form of ASK1 not phosphorylatable by AKT2, ASK1-S83A, prepared by converting Ser-83 of ASK1 to alanine. We also prepared ASK1-S83D, derived from mutating Ser-83 of ASK1 to aspartic acid, which mimics ASK1 phosphorylated by AKT2. A2780S cells were transfected with ASK1-S83A or ASK1-S83D, with or without constitutively active AKT2. Following cisplatin treatment, ASK1s were immunoprecipitated, and *in vitro* ASK1 kinase assays were performed using myelin basic protein as substrate. As shown in Fig. 3*E*, cisplatin significantly induced the kinase activity of both wild type ASK1 and nonphosphorylatable ASK1-S83A but not AKT2 phosphomimic ASK1-S83D. Expression of constitutively active AKT2 inhibited cisplatin-stimulated kinase activity of wild type ASK1 but not that of nonphosphorylatable ASK1-S83A. These results indicate that ASK1 kinase activity is negatively regulated by AKT2 through phosphorylation of ASK1 at Ser-83.

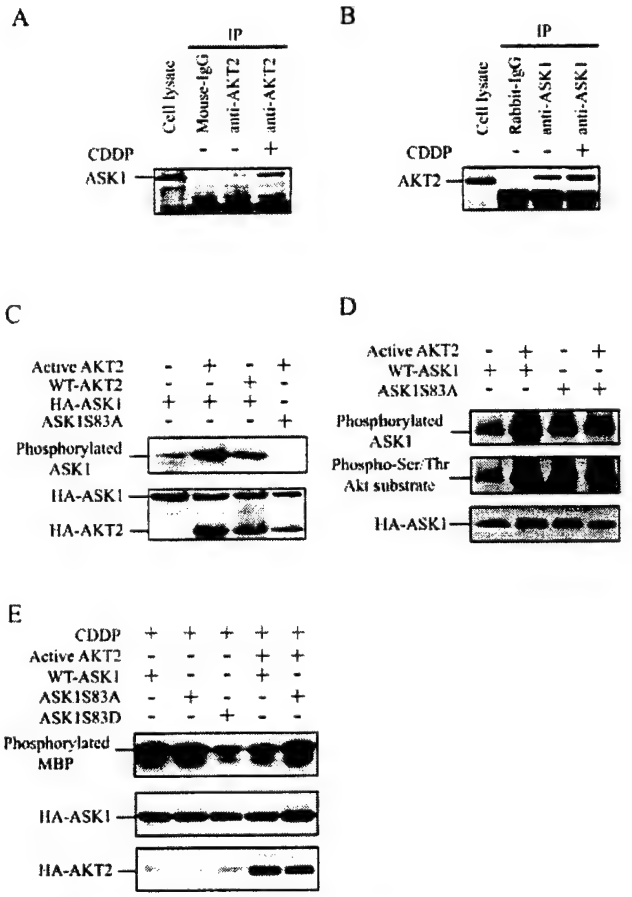

FIG. 3. **AKT2 interacts with and phosphorylates ASK1 and inhibits ASK1 kinase activity.** *A* and *B*, Western blot analyses of the immunoprecipitates prepared from A2780S cells treated with or without cisplatin. Immunoprecipitation was performed with anti-AKT2 and detected with anti-ASK1 antibody (*A*) and vice versa (*B*). *C*, *in vitro* kinase analysis of AKT2 immunoprecipitates derived from HEK293 cells that were transfected with indicated plasmids. Immunopurified HA-ASK1 or HA-ASK1-S83A was used as substrate (*top panel*). *The bottom panel* shows expression of transfected plasmids. *D*, *in vivo* [^{32}P]P_i labeling. HEK293 cells were transfected with indicated expression constructs, labeled with [^{32}P]P_i (0.5 mCi/ml), and immunoprecipitated with anti-HA antibody. The HA-ASK1 immunoprecipitates were separated in SDS-PAGE, blotted, and exposed to x-ray film (*top panel*). The membrane was then detected with anti-Akt substrate antibody (*middle panel*) and anti-HA antibody (*bottom panel*). *E*, *in vitro* ASK1 kinase analysis of the immunoprecipitates prepared from A2780S cells transfected with indicated plasmids and treated with cisplatin (20 μM) for 6 h. Myelin basic protein was used as substrate (*top panel*). Expression of transfected different forms of ASK1 and AKT2 was shown in the *second and third panels*.

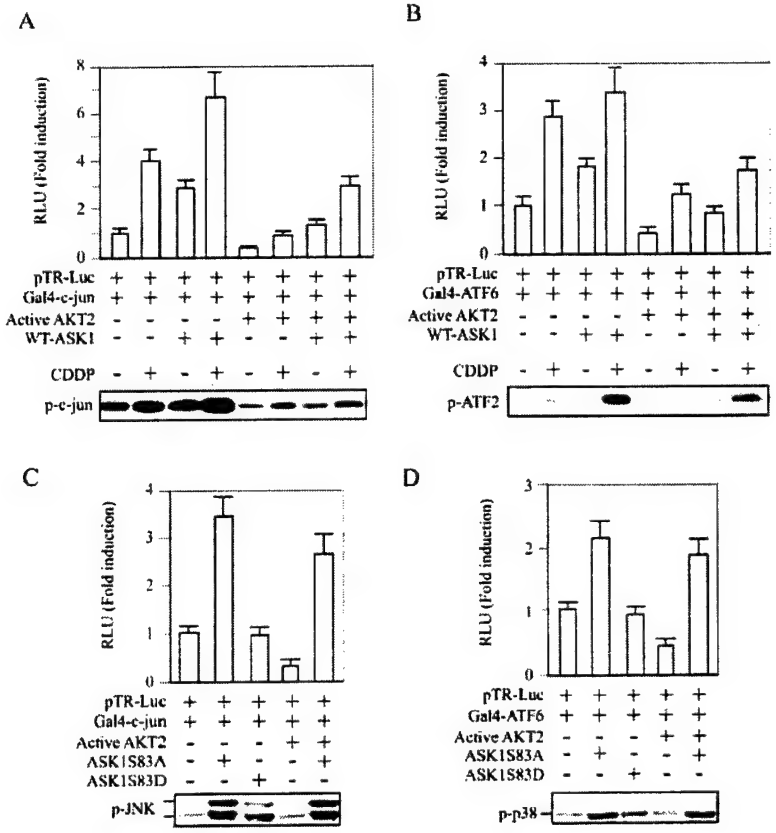

FIG. 4. **Activation of AKT2 inhibits ASK1- and/or cisplatin-induced JNK and p38 activation.** *A–D*, luciferase reporter assays. A2780S cells were transfected with indicated expression constructs and treated with or without cisplatin. Luciferase and β-galactosidase assays were performed, and the reporter activity was normalized by dividing luciferase activity with β-galactosidase. Each experiment was repeated three times. The *bottom panels* of *A* and *B* show the results obtained from *in vitro* JNK and p38 kinase assays using GST-c-Jun and ATF2 as substrates, respectively. The effects of AKT2 and its phosphorylation of ASK1 at Ser-83 on JNK and p38 activation were shown in the *bottom panels* of *C* and *D*.

AKT2 Inhibition of Cisplatin-stimulated JNK and p38 Is Mediated by Phosphorylation of ASK1 at Residue Ser-83—We next determined whether phosphorylation of ASK1 on Ser-83 by AKT2 is required for AKT2 inhibition of p38 and JNK, which are downstream from ASK1. Luciferase reporter assays were performed using Gal4-c-Jun/pTR-Luc (for JNK) and Gal4-ATF6/pTR-Luc (for p38) reporter systems. A2780S cells were transfected with ASK1, ASK1-S83A, ASK1-S83D, and/or Myr-AKT2, as well as pTR-Luc, Gal4-c-Jun, or Gal4-ATF6, and treated with or without cisplatin. Three independent experiments revealed that cisplatin induces Gal4-c-Jun or Gal4-ATF-regulated reporter activities. Further, *in vitro* JNK and p38 kinase analysis revealed that the phosphorylation of c-Jun and ATF2 was also stimulated by cisplatin treatment. These effects were enhanced by ectopic expression of wild type ASK1; however, they were inhibited by expression of constitutively active AKT2 (Fig. 4, *A* and *B*). Expression of nonphosphorylatable ASK1-S83A was also sufficient to induce the reporter activities and to attenuate the inhibitory action of constitutively active AKT2. In contrast, phosphomimic ASK1-S83D failed to stimulate the reporter activities (Fig. 4, *C* and *D*). Moreover, the effects of ASK1-S83A and ASK1-S83D on cisplatin-induced JNK and p38 activation were similar to their action on Gal4-c-Jun and Gal4-ATF6 reporters (Fig. 5A). Therefore, we conclude that AKT2 inhibits cisplatin-induced JNK and p38 via a phosphorylation of ASK1-dependent manner.

Cisplatin-induced Bax Conformational Change Is Regulated by AKT2 Phosphorylation of ASK1—Previous studies have shown that JNK is required for UV- and cisplatin-induced Bax conformational change (39). Our data demonstrate that ectopic expression of constitutively active AKT2 overrides cisplatin-induced ASK1/JNK/p38 activation and prevents formation of the active Bax conformation (see Figs. 1 and 2). To more directly probe the effect of AKT2 phosphorylation of ASK1 on Bax activation, we transfected A2780S cells with nonphosphorylatable and phosphomimic ASK1 and treated the cells with or without cisplatin. As revealed by immunoprecipitation and Western blot analyses, ectopic expression of nonphosphorylatable ASK1-S83A enhance cisplatin-dependent Bax conformational change, whereas ASK1-S83D, mimicking ASK1 phosphorylated by AKT2, inhibited cisplatin-induced Bax

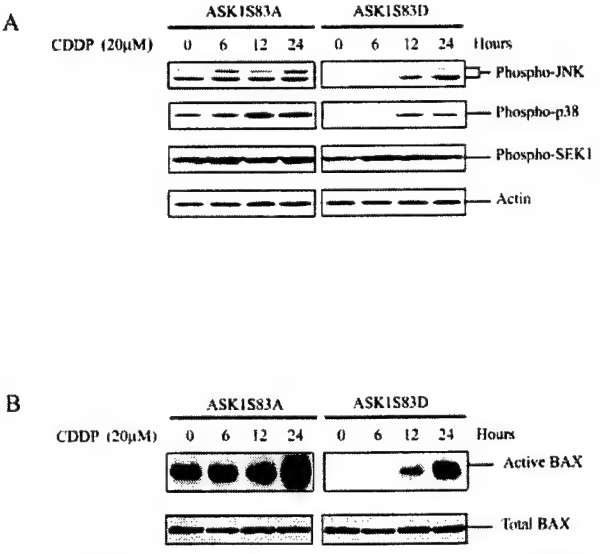

FIG. 5. **AKT2 phosphorylation of ASK1 at Ser-83 plays a critical role in cisplatin-induced JNK/p38 activation and Bax conformational change.** *A*, immunoblotting analysis of A2780S cells transfected with nonphosphorylatable and phosphomimic ASK1 prior to treatment with cisplatin. The blots were probed with the indicated antibodies. *B*, Western blot analysis. A2780S cells were transfected with indicated expression plasmids, treated with cisplatin, immunoprecipitated with anti-active Bax antibody, and detected with anti-total Bax antibody (*top panel*). Expression of Bax was shown in the *bottom panel*.

activation (Fig. 5*B versus* Fig. 1*B*). These data suggest that AKT2 inhibition of cisplatin-stimulated Bax conformational change is mediated at least to some extent by AKT2 phosphorylation of ASK1 at residue Ser-83.

Because JNK and p38 are downstream targets of ASK1, we next examined their roles in ASK1-stimulated Bax activation by using selective small molecule inhibitors of JNK and p38, JNK inhibitor II and SB 203580. As illustrated in Fig 6*A*, expression of ASK1 was sufficient to induce a Bax conformational change, and this effect was enhanced by cisplatin treatment. However, the conformational change of Bax induced by ASK1 and/or cisplatin was significantly diminished following treatment of cells with JNK inhibitor II (10 μM) and p38 inhibitor, SB 203580 (10 μM), suggesting that JNK and/or p38 mediate cisplatin-induced Bax activation. To probe the individual contributions of JNK and p38 in cisplatin-stimulated Bax activation, we further examined the effects of small molecule inhibitors of p38 and JNK and the expression of wild type and dominant negative forms of these kinases. A2780S cells were transfected with wild type or dominant negative JNK or p38, together with ASK1, and treated with or without cisplatin and/or inhibitor of JNK or p38. As shown in Fig. 6, *B* and *C*, expression of wild type JNK or p38 enhanced ASK1- and cisplatin-induced Bax activation, as expected. Furthermore, dominant negative JNK or a small molecule JNK inhibitor significantly decreased the Bax activation induced by cisplatin treatment or ectopic expression of ASK1 (Fig. 6*B*). We observed that only slight inhibition of the Bax activation was in the cells expressing dominant negative p38 or treated with p38 inhibitor (Fig. 6*C*). These results indicate that cisplatin- and/or ASK1-induced Bax activation is mediated primarily by JNK.

Inhibition of PI3K/AKT2 Pathway Sensitizes Cells to Cisplatin-induced Apoptosis—Because activated AKT2 reduces the cisplatin sensitivity of A2780S cells, we next examined the ability of inhibition of the PI3K/AKT2 pathway to sensitize cells to cisplatin-induced apoptosis. Cisplatin-resistant A2780CP and A2780S cells were transfected with dominant negative AKT2 or treated with PI3K inhibitor, LY294002, together with cisplatin. Tunel assay analyses revealed that either LY294002 or ectopic expression of dominant negative AKT2 enhanced cisplatin-induced apoptosis as compared with cells treated with cisplatin alone (Fig. 7, *A* and *C*). Accordingly, cleavage of caspase-3 and PARP was increased by treatment of cells with a combination of cisplatin with LY294002 or dominant negative-AKT2 (Fig. 7, *B* and *D*). To examine the role played by AKT2 phosphorylation of ASK1 in cisplatin-induced apoptosis, we transfected A2780S cells with ASK1-S83A, which is not phosphorylated by AKT2, ASK1-S83D, which mimics AKT2 phosphorylation, and then induced apoptosis with cisplatin. Notably, ectopic expression of ASK1-S83A significantly augmented cisplatin-induced apoptosis. In contrast, expression of ASK1-S83D conferred resistance to cisplatin (Fig. 7*E*). These data further indicate that PI3K/AKT2 promotes cell survival through phosphorylation and inhibition of ASK1 signaling.

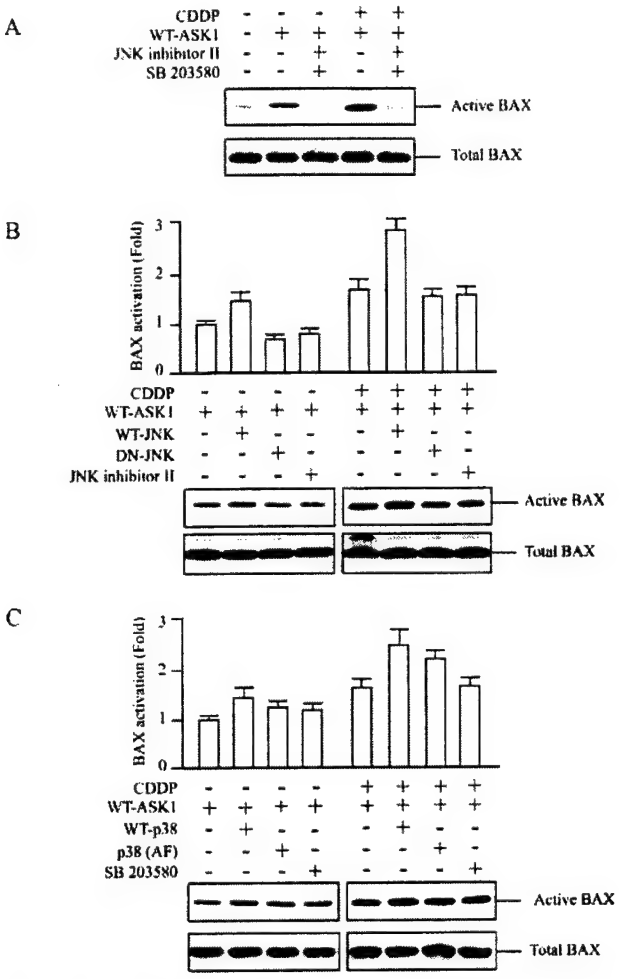

FIG. 6. **JNK and p38 mediate cisplatin- and ASK1-induced Bax conformational change.** *A*, Western blot analysis. A2780S cells were transfected with ASK1 and treated with JNK inhibitor II (10 μM) and SB 203580 (10 μM) for 1 h prior to addition of cisplatin. Following 16 h of the further treatment, Bax conformational change was examined as described above. *B* and *C*, immunoblotting analyses. A2780S cells were transfected with indicated plasmids and treated with indicated reagents. Bax conformational change was evaluated as described above. Both JNK inhibitor and dominant negative JNK exhibited more significant inhibitory effects on Bax activation than did p38 inhibitor and dominant negative p38 (AF). All the experiment was repeated three times.

DISCUSSION

We have demonstrated previously (18, 34) that AKT2 kinase is frequently elevated in human ovarian and breast cancers and that AKT2, like Akt1, exerts its anti-apoptotic function through phosphorylation of Bad (20). However, the biological role of AKT2 activation in human cancer and the mechanism of AKT2-induced cell survival in a chemotherapeutic setting have not been well documented. In this study, we show that activation of AKT2 significantly increases the resistance of ovarian cancer cells to cisplatin. AKT2 protects cells from cisplatin-induced apoptosis by inhibiting cisplatin-induced JNK/p38 activation and Bax conformational change. AKT2 mediates these effects through its interaction and phosphorylation of ASK1.

Cisplatin-induced JNK and p38 activations are required for its anti-tumor activity (22, 23). This activation has been shown to correlate with induction of apoptosis by cisplatin (22, 23). Moreover, studies using dominant negative mutants of JNK and p38 and specific pharmacological inhibitors have shown that activation of JNK and/or p38 is necessary for stress or chemotherapeutic drug-induced apoptosis (38, 40). Also, studies on fibroblasts with targeted disruptions of all the functional *Jnk* genes established an essential role for JNK in UV- and other stress-induced apoptosis (41). ASK1, an upstream regulator of JNK/p38, has also been shown to be induced by cisplatin (32). Furthermore, oxidative stress-induced ASK1 kinase activity is inhibited by Akt1 (42). Consistent with this, we demonstrate that activation of AKT2 inhibits cisplatin-induced JNK and p38 through direct interaction with and phosphorylation of ASK1 at serine 83. We also demonstrate that phosphorylation of ASK1 by AKT2 renders cells resistant to cisplatin.

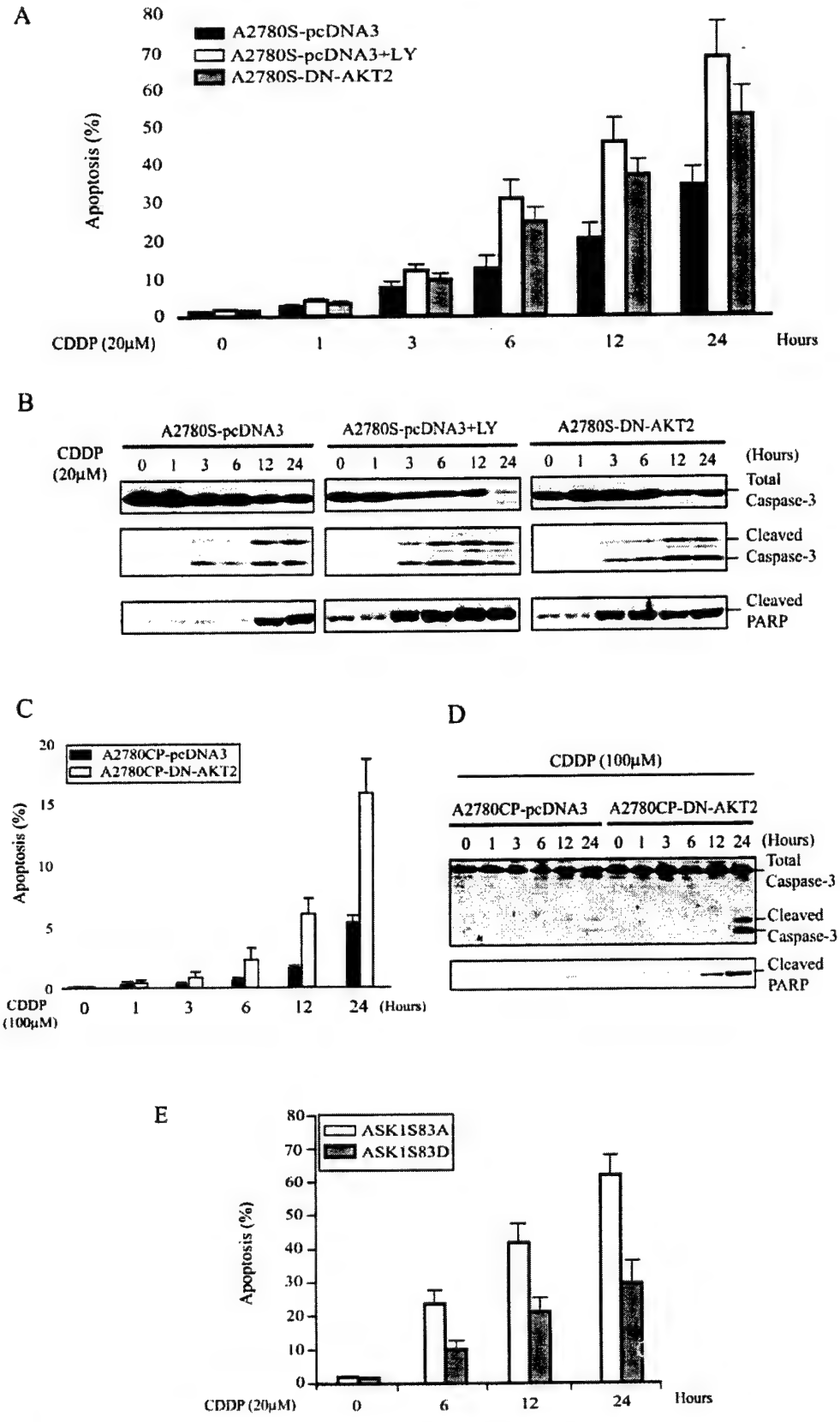

FIG. 7. **Inhibitions of PI3K/AKT2 and ASK1 phosphorylation sensitize cells to cisplatin-induced apoptosis.** *A*, Tunel assay. A2780S cells were transfected with dominant negative AKT2 or pcDNA3 vector and treated with cisplatin or cisplatin/LY294002. Apoptosis was examined and quantified after treatment for the indicated times. *B*, immunoblotting analysis of cell lysates prepared from cells treated as *A*. The blots were probed with indicated antibodies. *C* and *D*, cisplatin-resistant A2780CP cells transfected, treated, and analyzed as described in *A* and *B* except LY294002 treatment. *E*, Tunel assay. A2780S cells were transfected with indicated plasmids and treated with cisplatin. All the experiments were performed in triplicate.

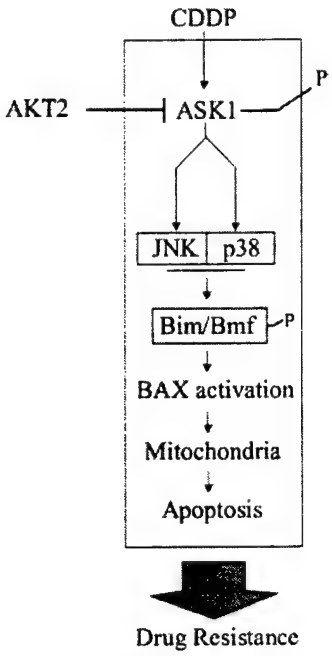

FIG. 8. **Schematic illustration of AKT2 regulation of ASK1/JNK/p38 and Bax.**

Besides the direct inhibition of ASK1, AKT2 could regulate JNK and p38 through other mechanisms. For example, NFκB-induced X chromosome-linked inhibitor of apoptosis and GADD45β down-regulate TNFα-induced JNK signaling (43, 44). We have demonstrated previously (21) that AKT2 inhibits UV- and TNFα-induced JNK and p38 by activation of the NFκB pathway (21). Therefore, we examined the possibility of AKT2 up-regulation of X chromosome-linked inhibitor of apoptosis and GADD45β. Western and Northern blot analyses, however, revealed no difference in X chromosome-linked inhibitor of apoptosis and GADD45β expression in A2780S cells transfected with constitutively active AKT2 or the control plasmid, pcDNA3 (data not shown). The possible reason is that cisplatin, unlike UV and TNFα, is incapable of inducing the NFκB pathway in A278S cells. In fact, our reporter assay revealed that cisplatin inhibits rather than activates NFκB activity in A2780S cells (data not shown).

In the present study, we observed that the ability of AKT2 to inhibit cisplatin-induced JNK/p38 was attenuated by nonphosphorylatable ASK1-S83A. Expression of phosphomimic ASK1-S83D alone was sufficient to inhibit JNK/p38 activation (Fig. 4). In addition, ASK1-S83D exhibited effects similar to that of constitutively active AKT2, *i.e.* rendered cells resistant to cisplatin, whereas ASK1-S83A sensitized cells to cisplatin-induced apoptosis (Fig. 7*E*). Thus, we conclude that AKT2 inhibition of cisplatin-stimulated JNK/p38 activation leading to cisplatin resistance is mediated by AKT2 phosphorylation/inhibition of ASK1.

It has been demonstrated that cisplatin-induced Bax conformational change is also important for cisplatin-stimulated apoptosis (45). Bax is a pro-apoptotic member of the Bcl2 family. Accumulated evidence shows that death signals, including cisplatin, induce a conformational change of Bax, leading to its mitochondrial translocation, oligomerization or cluster formation, and cytochrome *c* release (46, 47). Recent studies from Bax and/or Bak knock-out cells have shown that BH3-only proteins, such as tBid, Bad, Puma, and Bim, are required for inducing the activation of Bax and Bak by their direct interaction (48). Moreover, Akt has been shown to effectively inhibit Bax conformational change and contribute to chemoresistance (49). However, the mechanism by which Akt blocks Bax activation is poorly documented. We demonstrate in this report that ASK1 mediates at least in part cisplatin-induced Bax conformational change. Ectopic expression of constitutively active AKT2 attenuates cisplatin-induced Bax activation by phosphorylation and inhibition of ASK1. Downstream targets of ASK1, JNK, and p38, especially JNK, mediate AKT2 inhibition of Bax conformational change. These results are consistent with the recent findings obtained from a *Jnk*-deficient cell model (39).

Accumulated evidence shows that AKT2 plays a more significant role in human oncogenesis than AKT1 and AKT3. Frequent alterations of AKT2, but not AKT1 and AKT3, were detected in human cancers (18). Further, ectopic expression of AKT2, but not AKT1 and AKT3, leads to increased invasion and metastasis of human breast and ovarian cancer cells (50) and to malignant transformation of mouse fibroblasts (19). We observed in this study that A2780S cells expressing constitutively active AKT2 became cisplatin-resistant whereas expression of dominant negative AKT2 or treatment with PI3K inhibitor sensitized both cisplatin-sensitive (A2780S) and -resistant (A2780CP) ovarian cancer cells to cisplatin-induced apoptosis. Moreover, cisplatin-induced programmed cell death was enhanced by the expression of AKT2 nonphosphorylatable ASK1-S83A, whereas it is inhibited by phosphomimic ASK1-S83D. These data, therefore, indicate that activation of AKT2 contributes to cisplatin resistance by regulation of the ASK1/JNK/p38/Bax pathway and that the PI3K/AKT2/ASK1 cascade could be a critical therapeutic target for human cancer (Fig. 8).

A recent report (51) demonstrates that JNK and p38 phosphorylate BH3-only proapoptotic proteins Bim and Bmf, which was thought to mediate UV-induced apoptosis through a Bax-dependent mitochondrial apoptotic pathway (Fig. 8). Further investigation is required to determine the molecular mechanism by which ASK1/JNK/p38 regulates Bax activation in ovarian cancer cells, *i.e.* whether ASK1 and/or cisplatin induce Bim and Bmf phosphorylation and whether the phosphorylation is inhibited by PI3K/AKT2 pathway.

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ORIGINAL PAPER

Phosphatidylinositol-3-OH kinase/AKT and survivin pathways as critical targets for geranylgeranyltransferase I inhibitor-induced apoptosis

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Geranylgeranyltransferase I inhibitors (GGTIs) represent a new class of anticancer drugs. However, the mechanism by which GGTIs inhibit tumor cell growth is still unclear. Here, we demonstrate that GGTI-298 and GGTI-2166 induce apoptosis in both cisplatin-sensitive and -resistant human ovarian epithelial cancer cells by inhibition of PI3K/AKT and survivin pathways. Following GGTI-298 or GGTI-2166 treatment, kinase levels of PI3K and AKT were decreased and survivin expression was significantly reduced. Ectopic expression of constitutively active AKT2 and/or survivin significantly rescue human cancer cells from GGTI-298-induced apoptosis. Previous studies have shown that Akt mediates growth factor-induced survivin, whereas p53 inhibits survivin expression. However, constitutively active AKT2 failed to rescue the GGTIs downregulation of survivin. Further, GGTIs suppress survivin expression and induce programmed cell death in both wild-type p53 and p53-deficient ovarian cancer cell lines. These data indicate that GGTI-298 and GGTI-2166 induce apoptosis by targeting PI3K/AKT and survivin parallel pathways independent of p53. Owing to the fact that upregulation of Akt and survivin as well as inactivation of p53 are frequently associated with chemoresistance, GGTI could be valuable agents to overcome antitumor drug resistance.
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Keywords: Akt; PI3K; survivin; GGTI; apoptosis; ovarian cancer

Introduction

Geranylgeranyltransferase I and farnesyltransferase inhibitors (GGTIs and FTIs) represent a new class of anticancer drugs (Sebti and Hamilton, 2000). These compounds were originally designed to block lipid post-translational modification of oncogenic Ras, which is essential for its function (Reiss *et al*., 1990; Kohl *et al*., 1993). Prenylation of small G proteins such as Ras, Rho, and Rac is critical to their cellular localization and function. Two types of prenyl transferases, farnesyltransferase and geranylgeranyltransferase (GGTase), have been shown to catalyse protein prenylation. FTase catalyses the transfer of farnesyl from farnesylpyrophosphate to cysteine at the carboxyl terminus of proteins ending in CAAX, where C is cysteine and A is an aliphatic amino acid, and X is methionine, serine, cysteine or glutamine. GGTase I, on the other hand, transfers geranylgeranyl from geranylgeranylpyrophosphate to CAAX terminal sequences, where X is leucine or isoleucine. We have developed CAAX peptidomimetics such as GGTI-298 and FTI-277 as highly selective inhibitors of GGTase I and FTase, respectively (Sebti and Hamilton, 1997). FTI-277 blocks potently oncogenic H-Ras processing and signaling. However, inhibition of the processing of K-Ras, the most prevalent form of mutated Ras in human tumors, becomes geranylgeranylated by GGTase I when FTase is inhibited. Therefore, both FTI-277 and GGTI-298 are required for inhibition of K-Ras processing in human tumor (Sebti and Hamilton, 1997). Several reports suggested that RhoB is a critical target for antitumor activity of FTIs (Du *et al*., 1999; Prendergast, 2001). However, this remains conversable and other studies have provided evidences against inhibition of Rho-B farnesylation as a mechanism by which FTIs inhibit tumor cell survival and growth (Chen *et al*., 2000).

Inhibitor of apoptosis proteins (IAPs) represent a conserved gene family that protects against programmed cell death induced by a variety of apoptotic stimuli (Deveraux and Reed, 1999). IAPs contain at least one BIR (baculovirus IAP repeat) domain that binds to caspases 3, 7, and 9 to inhibit their activities. Survivin is the smallest known IAP family protein and contains a single BIR domain with which it binds caspases and prevents caspase-induced apoptosis (Altieri, 2003). In addition, survivin also plays an important role in cell cycle control (Reed, 2001). Altered expression of survivin appears to be a common event associated with

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the pathogenesis of human cancer; survivin is overexpressed in many transformed cell lines and in common cancers, such as those of the ovary, lung, colon, liver, prostate, and breast (Reed, 2001; Altieri, 2003). Reduced survivin expression causes apoptosis and sensitization to anticancer drugs, suggesting that survivin expression is important for cell survival or chemoresistance of certain carcinomas (Tran *et al*., 2002; Altieri, 2003).

Phosphatidylinositol-3-OH kinase/Akt is another major cell survival pathway that has been recently extensively studied (Brazil *et al*., 2002). PI3K is a heterodimer composed of a p85-regulatory and a p110-catalytic subunit and converts the plasma membrane lipid phosphatidylinositol-4-phosphate [PI(4)P1] and phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] to phosphatidylinositol-3,4,-bisphosphate [PI(3,4)P2] and phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3]. Pleckstrin-homology (PH) domain-containing proteins, including Akt, accumulate at sites of PI3K activation by directly binding to PI(3,4)P2 and PI(3,4,5)P3. Akt (also known as PKB) represents a subfamily of the serine/threonine kinases. Three members of this family, including AKT1, AKT2, and AKT3, have been identified so far. Akt is activated by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress in a PI3K-dependent manner (Franke *et al*., 1995; Datta *et al*., 1999). Several downstream targets of Akt, each of which contains the Akt phosphorylation consensus sequence R–X–R–X–X–S/T–F/L, have been identified (Datta *et al*., 1999), pointing to the possible mechanisms by which Akt promotes cell survival and blocks apoptosis. Akt phosphorylates the proapoptotic proteins BAD, caspase-9, and transcription factor FKHRL1, resulting in reduced binding of BAD to Bcl-X_L and inhibition of caspase-9 protease activity and Fas ligand transcription (Datta *et al*., 1999). Moreover, alteration of Akt, especially AKT2, have been frequently detected in human malignancy. Overexpression/activation of PI3K and/or Akt renders cancer cells resistant to conventional chemotherapeutic drugs (Cheng *et al*., 2002; Clark *et al*., 2002). It has also been shown that inactivation of PTEN and p53 results in constitutive activation of Akt pathway. PTEN mutation is related to loss of its lipid phosphatase activity, and thus, it is unable to convert PI(3,4,5)P3 to PI(4,5)P2 (Datta *et al*., 1999). p53 transcription factor has recently been found to bind to the promoters of PTEN and p110α to induce PTEN and inhibit p110α transcription. Therefore, mutations of p53 result in downregulation of PTEN and upregulation of p110α leading to activation of Akt (Stambolic *et al*., 2001; Singh *et al*., 2002).

In the present study, we demonstrate that GGTI-298 and GGTI2166 target PI3K/AKT2 and survivin pathways leading to programmed cell death in cisplatin-sensitive and -resistant human ovarian cancer cells via a p53-independent mechanism. Moreover, AKT1 activation was also inhibited by GGTI-298 and GGTI-2166. As AKT2, but not AKT1, is frequently altered in human cancer (Cheng *et al*., 1992; 1996; Yuan *et al*., 2000; Arboleda *et al*., 2003), we primarily focused our study on AKT2.

Results and discussion

GGTIs inhibit AKT2 and induces apoptosis in cisplatin-sensitive and -resistant human ovarian cancer cells

We have previously demonstrated that GGTI-298 arrests NIH 3T3 cells and lung cancer cells at G1 phase by upregulation of $p21^{WAF/CIP1}$ and hypophosphorylation of RB (Adnane *et al*., 1998; Sun *et al*., 1999a, b). We have also documented that GGTIs enhance the ability of FTIs to induce apoptosis in drug-resistant myeloma (Bolick *et al*., 2003) as well as synergize with other anticancer drugs such as cisplatin, taxol, and gemcitabine to inhibit human lung cancer cell growth in nude mice (Sun *et al*., 1999a, b). These results implicate the role of geranylgeranylated proteins in cell survival control, yet the involved mechanisms for inhibition of tumor growth and induction of apoptosis still remain unclear. Our previous studies showed that constitutively active H-Ras significantly activates PI3K/AKT2 and that the farnesyltransferase inhibitor, FTI-277, suppresses the PI3K/AKT2 pathway leading to cell death in human cancer cell lines (Liu *et al*., 1998; Jiang *et al*., 2000). These studies prompted us to examine the possible involvement of the PI3K/Akt pathway in GGTI antitumor activity. A cisplatin-sensitive (A2780S) and a cisplatin-resistant (A2780CP) ovarian cancer cell lines were treated with GGTI-298 (15 μM) or GGTI-2166 (20 μM) in DMEM supplemented with 10% FBS for 0, 12, 24, 36, and 48 h. Apoptosis and Akt activation were analysed by the Tunel assay and Western blot. Following GGTI-298 or GGTI-2166 treatment, both cisplatin-sensitive A2780S and cisplatin-resistant A2780CP cells underwent programmed cell death. Apoptotic cells reached approximately 70–80% after 36 h of treatment without significant difference between these two cell lines (Figure 1a and data not shown), indicating that GGTI-298 and GGTI-2166 are able to overcome cisplatin resistance in human ovarian cancer cells.

Immunoblotting analysis of AKT1 and AKT2 immunoprecipitates with phosphor-Akt-Ser473 antibody revealed that GGTI-298 and GGTI-2166 inhibit phosphorylation of AKT1 and AKT2 after 12 h of treatment in both cisplatin-sensitive and -resistant cell lines. However, total AKT1 and AKT2 protein levels remained unchanged (Figure 1b and data not shown). These results suggest that GGTI may either directly or indirectly target Akt signal transduction pathway to induce apoptosis.

GGTIs target a geranylgeranylated protein(s) upstream of PI3K/AKT2 pathway

To demonstrate that GGTI-298 and GGTI-2166 actually suppress AKT2 kinase, A2780S cells were treated with or without EGF (50 ng/ml) for 15 min following

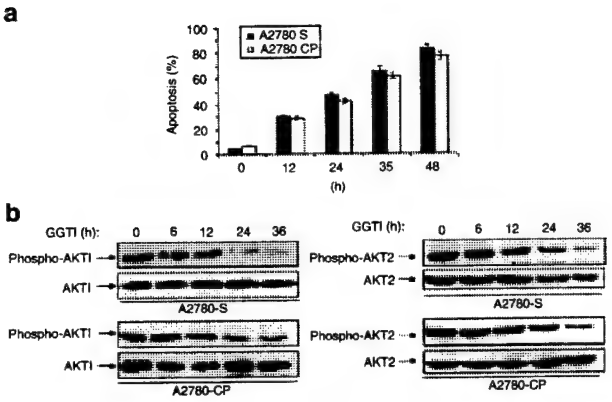

Figure 1 GGTI-298 inhibits Akt activation and induces apoptosis in cisplatin-sensitive and -resistant ovarian cancer cells. (**a**) Tunel assay. Cisplatin-sensitive A2780-S and cisplatin-resistant A2780-CP cells were cultured in DMEM supplemented with 10% FBS and treated with GGTI-298 (15 μM) for the indicated time. Apoptotic cells were detected with the Tunel assay and quantified. (**b**) Western blot analyses of the AKT1 (left) and AKT2 (right) immunoprecipitates prepared from A2780S and A2780CP cells following GGTI-298 treatment. The blots were detected with anti-phospho-Akt-Ser473 (panels 1 and 3), -AKT1 and -AKT2 (panels 2 and 4) antibodies. All the experiments were repeated three times

treatment with GGTI-298 or GGTI-2166 for 12 h. *In vitro* kinase assays were then performed on AKT2 immunoprecipitates as described under Experimental procedures. As illustrated in Figure 2a, EGF-induced AKT2 kinase activity was abrogated by GGTI-298 treatment. As PI3K is an upstream activator of AKT2, we next examined whether GGTI-298 inhibits PI3K activity. Following GGTI-298 treatment and EGF stimulation as described above for AKT2 kinase assay, A2780S and A2780CP cells were immunoprecipitated with anti-pan-p85 antibody. PI3K activity was examined by *in vitro* kinase analysis of the immunoprecipitates using PI(4,5)P2 as a substrate. GGTI-298 attenuated EGF-stimulated PI3K activation (Figure 2c). However, GGTI-298 does not directly inhibit PI3K and AKT2 activities as determined by adding GGTI-298 to the kinase reaction *in vitro* (Figure 2b and d). In addition, GGTI-2166 exhibits the same effects on PI3K/Akt activation as GGTI-298 (data not

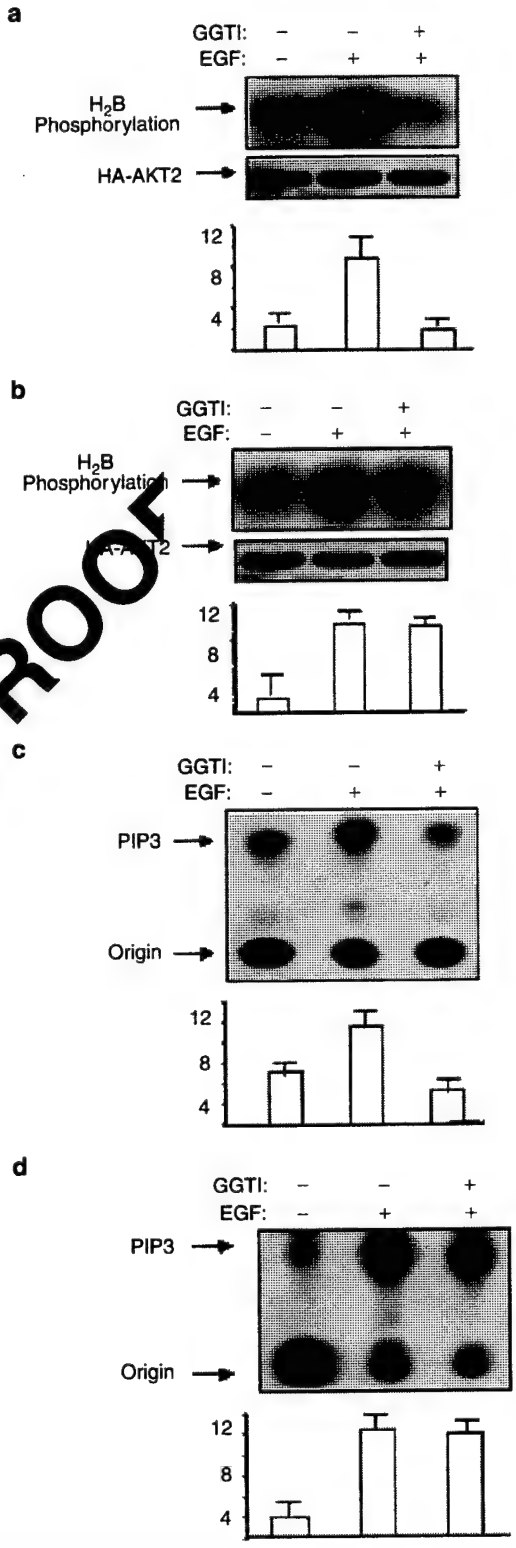

Figure 2 GGTI-298 inhibits PI3K and AKT2 activation. (**a**) *In vitro* kinase assay of the HA-AKT2 immunoprecipitates prepared from A2780S cells. After serum starvation overnight, the cells were treated with or without GGTI-298 for 12 h prior to EGF (50 ng/ml) stimulation for 5 min. Immunoprecipitation was performed with anti-AKT2 antibody and subjected to *in vitro* kinase assay using histone H2B as substrate. (**b**) GGTI-298 does not directly inhibit EGF-induced PI3K activation. After serum starvation and stimulation with EGF, GGTI-298 (15 μM) was directly added into AKT2 kinase reaction. Following incubation for 30 min, the reactions were separated on SDS–PAGE gel and exposed to the film. (**c**) *In vitro* PI3K assay of the anti-p85 immunoprecipitates prepared from A2780CP cells. Following serum starvation overnight, the cells were treated with or without GGTI-298 for 12 h prior to EGF stimulation for 15 min. (**d**) GGTI-298 does not directly inhibit EGF-induced PI3K activation. *In vitro* PI3K assay of the PI3K immunoprecipitates derived from A2780CP cells. After serum starvation and stimulation, GGTI-298 (15 μM) was directly added to the kinase reaction. Quantification of AKT2 and PI3K activity from three repeated experiments is shown in bottom panels (**a–d**)

shown). These data imply that GGTI-298 and GGTI-2166 are not direct inhibitors of PI3K and AKT2 but rather target a geranylgeranylated protein(s) upstream of PI3K/AKT2 pathway.

Constitutively active AKT2 partially rescues A2780S cells from GGTI-induced apoptosis

We reasoned that if GGTI-298 and GGTI-2166 inhibit a geranylgeranylated protein upstream of PI3K/AKT2, then constitutively active AKT2 should overcome GGTIs-induced apoptosis. A constitutively active AKT2 expression construct (HA-Myr-*AKT2*) or pcDNA3 vector alone was stably transfected into A2780S cells. Western blot analysis with anti-HA antibody revealed expression of HA-Myr-AKT2 in the transfectants (Figure 3a). After treatment with GGTI-298 (15 μM) or GGTI-2166 (20 μM) for different times in the presence of 10% FBS, apoptotic cells were observed in pcDNA3- and Myr-AKT2-transfected A2780S cells. The percentages of apoptotic cells in pcDNA3-transfected A2780S cells increased from 8% at time 0 to 80% after 48 h of treatment with GGTI-298 (Figure 3b). These percentages are very similar to those reported in Figure 1a for nontransfected parental A2780S cells. In contrast, GGTI-298 induced apoptosis by 40% at time 48 h of treatment in cells transfected with constitutively activated AKT2. Similar effects were observed in the cells treated with GGTI-2166 (data not shown). Therefore, constitutively active AKT2 only partially rescues A2780S cells from GGTI-induced apoptosis (Figure 3b), indicating that other cell survival signal molecule(s) must be targeted by GGTI-298 and GGTI-2166 besides PI3K/Akt pathway.

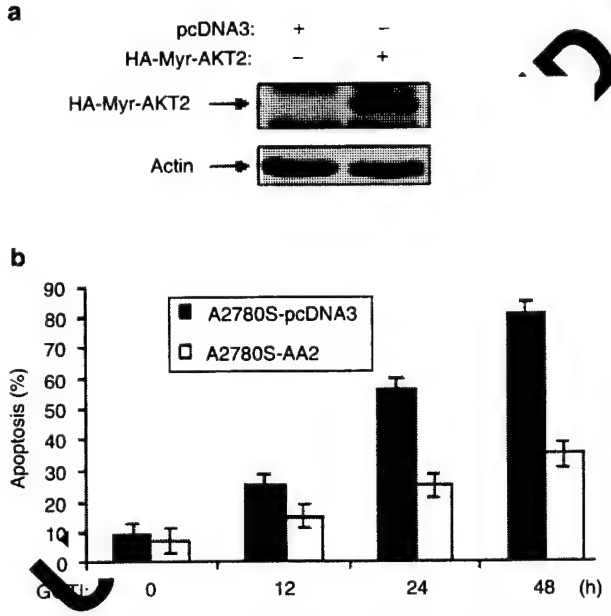

Figure 3 A constitutively activated form of AKT2 partially rescues A2780S cells from GGTI-298-induced apoptosis. (**a**) A2780S cells were stably transfected with constitutively active AKT2 (Myr-AKT2, AA2). Western blot analysis with anti-HA antibody revealed expression of transfected HA-Myr-AKT2 in a clonal cell line (upper panel). Bottom panel shows equal loading. (**b**) Tunel assay. After treatment of A2780S-pcDNA3 and A2780S-AA2 cells with GGTI-298 for the indicated times, apoptotic cells were detected and quantified from three independent experiments

GGTIs downregulate the IAP family protein survivin

Numerous studies have shown that IAP family proteins play a critical role in cell survival (Deveraux and Reed, 1999; Reed, 2001; Tran *et al*., 2002; Altieri, 2003). Among the members of IAP family, only survivin is frequently overexpressed in human cancer including ovarian carcinoma and ectopic expression of survivin renders ovarian cancer cells resistant to taxol (Zaffaroni *et al*., 2002). Thus, we next examined whether GGTI-298 or GGTI2166 target survivin to induce apoptosis in ovarian cancer cells. A2780S cells, in which survivin is highly expressed, were treated with GGTI-298 or GGTI-2166 for different times. Western and Northern blot analyses revealed that both protein and mRNA levels of survivin were significantly reduced following GGTI-298 or GGTI-2166 treatment (Figure 4a and data not shown). To further examine the importance of survivin in GGTIs [illegible]ptotic activity, A2780S cells were stably transfected with Myc-tagged survivin. Again, the cells transfected with pcDNA3 vector alone were used as control. Expression of transfected Myc-survivin was confirmed by immunoblotting analysis with anti-Myc antibody (Figure 4c). Following administration of GGTI-298 or GGTI-2166 at various lengths of time, apoptotic cells were detected by the Tunel assay and quantified. Both GGTI-298 and GGTI-2166 increased apoptosis from 8 and 10% at time 0 to 70 and 80% at time 24 h in A2780S-pcDNA3 cells, respectively. In survivin-expressing cells, both inhibitors induced apoptosis to only 30% after 24 h of treatment (Figure 4d and data not shown). Thus, ectopic expression of survivin rescues the cells from GGTI-induced apoptosis but only partially, implying that survivin is another target of GGTIs in addition to PI3K/AKT2. Further, A2780S cells were stably contransfected with constitutively active AKT2 and survivin (Figure 4c) and treated with either GGTI-298 (15 μM) or GGTI-2166 (20 μM). The Tunel assay analysis revealed that cells expressing both myr-AKT2 and survivin became dramatically resistant to GGTIs treatment (Figure 4d), indicating that AKT2 and survivin are critical targets of GGTIs at least in A2780S ovarian cancer cells.

GGTIs inhibits survivin via a p53-independent pathway

Previous investigations have demonstrated that p53 represses survivin expression through inhibiting its transcription (Hoffman *et al*., 2002; Mirza *et al*., 2002). To determine whether GGTIs' suppression of survivin expression depends on p53, we evaluated the effects of GGTIs on survivin expression in A2780CP cells that carry p53 mutation (Sasaki *et al*., 2000). A2780CP cells were cultured in DMEM supplemented with 10% FBS and treated with GGTI-298 or GGTI-

2166 for different times. The expression of survivin was evaluated by Western and Northern blot analyses. Both protein and mRNA levels of survivin were inhibited by GGTI-298 and GGTI2166 treatment in A2780CP cells (Figure 4b and data not shown). Quantification analysis showed that GGTI-inhibited survivin expression was similar in A2780CP that contain mutant p53 and A2780S cells that express wild-type p53 (Figure 4a and b). To further define the effects of p53 on GGTIs' suppression of survivin expression, A2780CP cells were stably transfected with HA-tagged wild-type p53 and pcDNA3 vector alone, as a control. Figure 5a shows that transfected p53 expresses and is functional reflected by elevated level of $p21^{WAF1}$ and restoration of A2780CP cells sensitive to cisplatin treatment. Immunoblotting

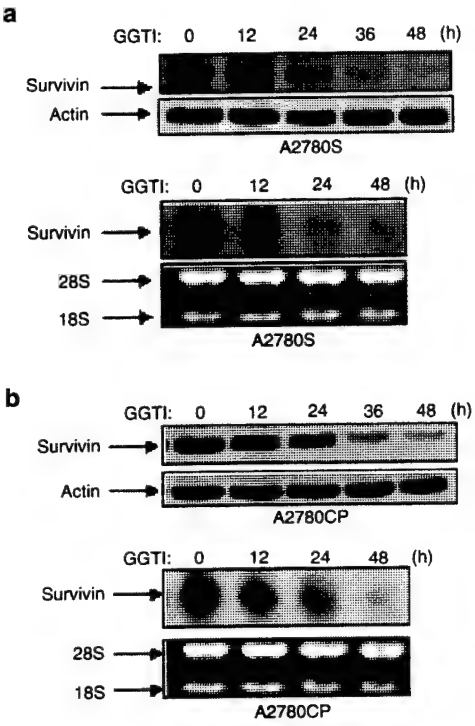

Figure 4 GGTI-298 inhibits expression of survivin independent of p53 pathway. (**a** and **b**) Western (upper panels) and Northern (lower panels) analyses of expression of survivin in A2780S (wild-type p53) and A2780CP (mutant p53) cells treated with GGTI-298 at indicated time. Northern blot analysis with [^{32}P]dCTP-labeled survivin cDNA probe (upper). Equal loading of total RNA was shown in bottom panel. (**c**) Immunoblotting analysis of expression of transfected Myc-survivin and HA-myr-AKT2 in A2780S cells with anti-Myc (upper) and anti-HA (bottom) antibodies. (**d**) Tunel assay. Following treatment of A2780S-pcDNA3, A2780S-survivin and A2780S-survivin/myr-AKT2 cells with GGTI-298 at indicated time, apoptotic cells were detected with the Tunel assay and quantified

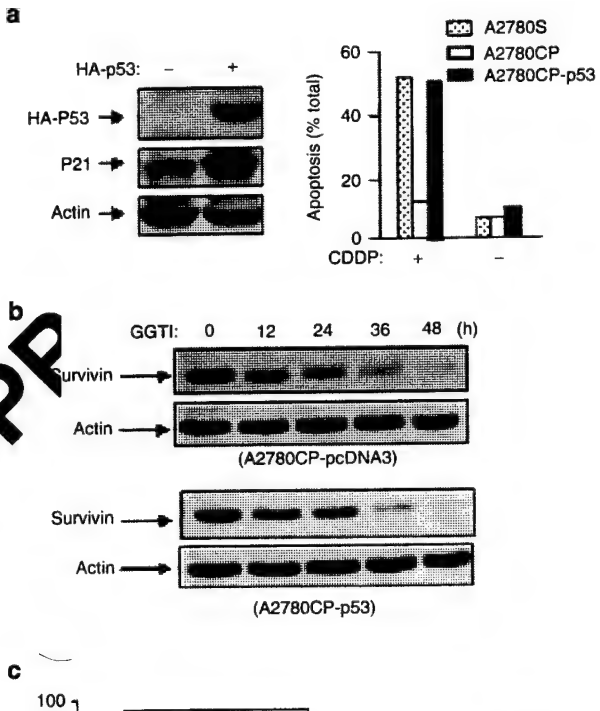

Figure 5 Ectopic expression of p53 did not affect GGTI action. (**a**) Immunoblotting analysis of expression of transfected wild-type HA-p53 in A2780CP cells with anti-HA (top) and anti-p21 (middle) antibodies. The bottom panel showed equal loading. (**b**) Immunoblotting analysis of survivin expression in pcDNA3- (upper panels) and HA-p53-transfected (bottom panels) A2780CP cells. (**c**) Reintroduction of wild-type p53 into A2780CP cells did not sensitize the cells to GGTI-298-induced apoptosis. Following GGTI-298 treatment at indicated time, apoptotic cells were detected and quantified from three independent experiments

analysis showed that reintroduction of wild-type p53 into A2780CP cells did not have significant effects on the ability of GGTIs to inhibit survivin expression as compared to pcDNA3-transfected A2780CP cells (Figure 5b). These results indicate that GGTIs suppression of survivin is independent of p53 pathway.

Previous studies have shown that re-expression of wild-type p53 sensitizes A2780CP cells to cisplatin-induced apoptosis (Song *et al*., 1997; Sasaki *et al*., 2000). Therefore, we next examined whether ectopic expression of wild-type p53 sensitizes A2780CP cells to GGTI-stimulated cell death. The Tunel assay revealed that the levels of GGTI-induced apoptosis were the same in A2780CP-p53, A2780CP-pcDNA3 as well as A2780S cells (Figures 5c and 1a). We have previously shown GGTI-298-mediated transcriptional upregulation of $p21^{WAF1/CIP1}$ is also independent of p53 (Adnane *et al*., 1998). This further supports the notion that the mechanism of GGTIs antitumor activity does not involve the p53 pathway. Since mutant p53 is a major contributor to anticancer drug resistance and since GGTIs can overcome this resistance at least in the case of cisplatin, combination of GGTIs with these agents has a potential for cancer treatment.

GGTIs attenuated AKT2-induced survivin expression and promoter activity

Recent studies have shown that PI3K/Akt pathway mediates IGF1- and VEGF-upregulation of survivin protein in multiple myeloma and endothelial cells (Papapetropoulos *et al*., 2000; Mitsiades *et al*., 2002). However, the underlying molecular mechanism has not been well documented. As GGTI inhibits PI3K/AKT activation as well as survivin expression at the transcription level, we reasoned that activation of AKT2 could induce survivin transcription. To this end, Northern blot analysis of A2780S cells transfected with constitutively active AKT2 revealed that expression of survivin was induced by AKT2 in a dose-dependent manner (Figure 6a). Further, a luciferase activity assay was carried out with HEK293 cells transfected with pGL3-survivin-Luc reporter, constitutively active AKT2 and β-galactosidase. Triple experiments showed that ectopic expression of constitutively active AKT2 stimulated survivin promoter activity (Figure 6b). These data indicate that AKT2 upregulates survivin by inducing its promoter activity. It has been demonstrated that survivin promoter contains a NFB-binding site and is induced by NFB pathway (Deveraux and Reed, 1999; Mitsiades *et al*., 2002). We and others have shown that AKT1 and AKT2 activate the NFκB pathway through

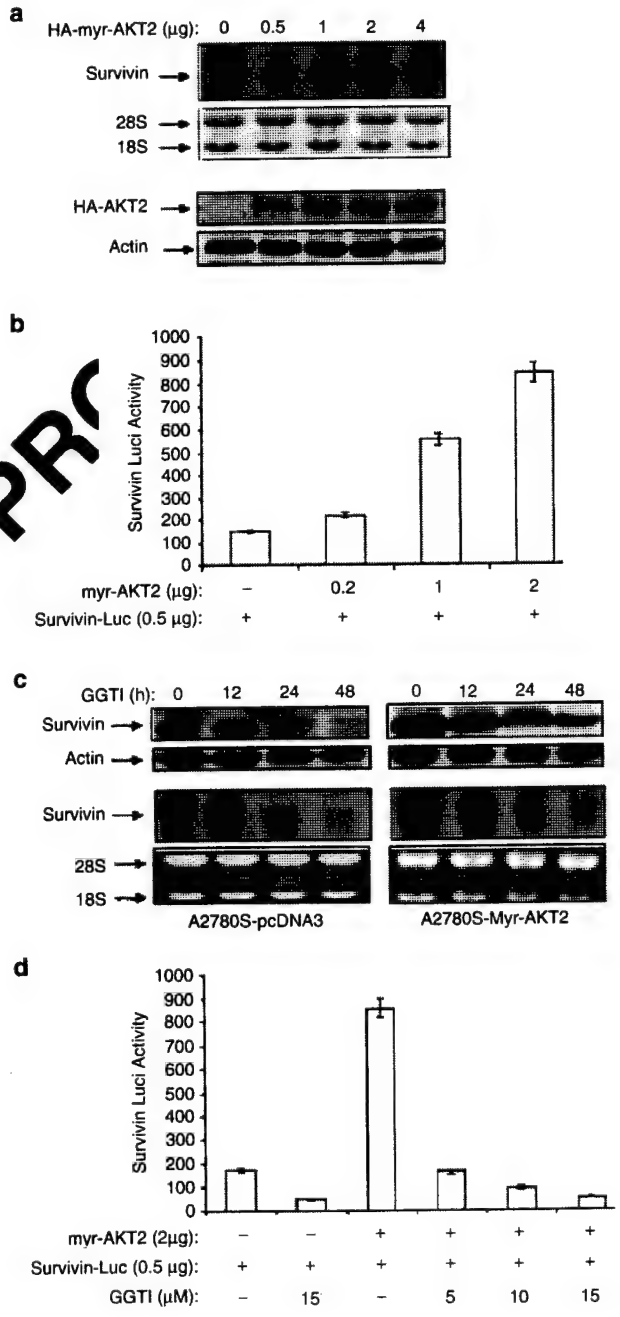

Figure 6 Constitutively active AKT induces survivin transcription and promoter activity; AKT failed to rescue GGTI-down-regulated survivin. (**a**) Northern blot analysis of A2780S cells transfected with indicated amount of constitutively active AKT2. The blot was probed with [^{32}P]dCTP-labeled survivin cDNA (upper panel). Equal loading was shown in panel 2. Expression of transfected constitutively active AKT2 was detected with anti-HA antibody (panel 3). The same blot was reprobed with antiactin antibody (bottom panel). (**b**) Luciferase reporter assay. HEK293 cells were transfected with indicated plasmids. After 36 h of the transfection, luciferase and β-galactosidase assays were performed and the reporter activity was normalized by dividing luciferase activity with β-galactosidase. (**c**) Western (panels 1 and 2) and Northern (panels 3 and 4) blot analyses of pcDNA3- and constitutively active AKT2-trnasfected A2780S cells following treatment with GGTI-298 at indicated time. Western blots were detected with antisurvivin (upper) and antiactin antibodies (panel 2). Northern blots were probed with [^{32}P]dCTP-labeled survivin (panel 3). Equal RNA loading was shown in bottom panels. The luciferase reporter assay was performed as described in (**b**), except the cells were treated with indicated concentrations of GGTI-298 for 6 h prior to assay for luciferase and β-galactosidase activity. Each experiment was repeated three times

interaction and phosphorylation of IKKα and Cot/Tpl2 (Ozes *et al*., 1999; Madrid *et al*., 2000; Kane *et al*., 2002; Yuan *et al*., 2002). Therefore, AKT2-induced survivin transcription could be mediated by activation of this pathway.

Since AKT2 upregulates survivin and GGTIs repress survivin expression and AKT2 activity, one possible mechanism by which GGTIs could repress survivin is through inhibition of PI3K/AKT2. To test this hypothesis, constitutively active AKT2- and pcDNA3-stably transfected A2780S cells were treated with GGTI-298 or GGTI-2166. Following the treatment for 12, 24, and 48 h, expression of survivin was examined by Western and Northern blot analyses. As shown in Figure 6c, both basal protein and mRNA levels of survivin were higher in A2780S-Myr-AKT2 cells as compared to A2780S transfected with pcDNA3 vector alone. However, declining rate of the survivin induced by GGTIs was essentially the same between constitutively active AKT2- and pcDNA3-transfected A2780S cells. Moreover, the luciferase reporter assay showed that constitutively active AKT2-stimulated survivin promoter activity was also attenuated by GGTIs treatment. Even the basal levels of survivin promoter activity were significantly inhibited by GGTI-298 or GGTI-2166 (Figure 6d and data not shown). As GGTI-298 and GGTI-2166 are not direct AKT2 inhibitor (Figure 2c), we conclude that GGTIs repress survivin by targeting other molecule(s), which bypasses AKT2 but is capable of blocking AKT2-induced survivin transcription. Moreover, these data also indicate that GGTIs induce apoptosis in human ovarian cancer cells by inhibition of survivin and PI3K/AKT2 parallel pathways.

Effects of FTI and/or GGTI on apoptosis, AKT activity, and survivin expression

To examine whether GGTI antitumor activity is mediated by decrease in protein geranylgeranylation or a compensatory increase in protein farnesylation, we cotreated A2780S cells with FTI and GGTI. The cells treated with FTI and GGTI alone were used as controls. Triple experiments revealed that FTI induces apoptosis at much lesser extent than GGTI. The apoptosis induced by cotreatment with GGTI and FTI is higher than that of either GGTI or FTI alone; however, FTI did not exhibit dramatic effects on GGTI-induced apoptosis in A2780S cells (Figure 7a). Immunoblotting analysis showed that GGTase I substrate Rap1 and of FTIase substrate HDJ2 were inhibited by GGTI-298 and FTI-277 in A2780S cells, respectively (Figure 7b). Moreover, GGTI-downregulated survivin was not affected by FTI treatment, even though phosphorylation level of Akt was inhibited by GGTI/FTI at higher degree as compared to GGTI or FTI alone (Figure 7c). These data suggest that FTI and GGTI have no significant synergistic inhibitory effects on cell survival and survivin expression in A2780S cells and that GGTI treatment did not result in a compensatory increase in protein farnesylation.

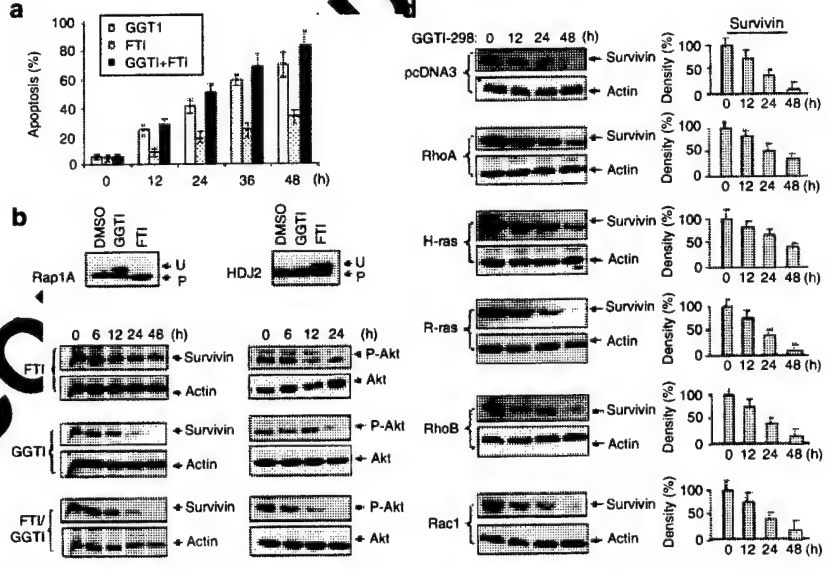

Figure 7 Effects of FTI and GGTI on cell survival, Akt activation and expression of survivin. (**a**) Tunel assay. A2780S cells were treated with GGTI-298 (15 μM) together with FTI-277 (20 μM) and GGTI or FTI alone. After treatment for indicated time, apoptotic cells were detected with Tunel assay. (**b**) A2780S cells were treated as panel a and subjected to Western blot analysis with anti-Rap1 and -HDJ2 antibodies. *U* and *P* designate unprocessed and processed forms of Rap1 and HDJ2. (**c**) Immunoblotting analysis of A2780S cells treated as described in panel a with indicated antibodies. (**d**) A2780S cell were transiently transfected with indicated plasmids using LipofactAmine Plus. Approximately 70% transfection efficiency was achieved using transfection of EGFP-C2 vector as an indicator. After 36 h of transfection, cells were treated with GGTI-298 (15 μM) and then immunoblotted with indicated antibodies (left panels). Right panels show the quantification of survivin protein levels from three independent experiments

As GGTI was originally designed to inhibit small G-proteins, we next examined whether ectopic expression of small G-protein(s) over-rides GGTI-dowregulated survivin. A2780S cells were individually transfected with expression constructs of v-H-Ras, R-Ras, Rac, RhoB, and RhoA. After 36 h of transfection, the cells were treated with GGTI-298 for different time, expression of survivin was analysed by Western blot. As shown in Figure 7d, survivin was significantly declined in GGTI-298-treated pcDNA3-transfected cells. However, none of small G-proteins examined has dramatic protection from GGTI downregulation of survivin even though H-ras and RhoA slightly inhibit GGTI-induced survivin declining rate. These data suggest that these small G-proteins do not seem to be the targets of GGTI to downregulate survivin in A2780S cells even though Akt has been shown to be activated by some of them (Datta *et al*., 1996; Liu *et al*., 1998).

In summary, the data presented here demonstrate for the first time that GGTI-298 and GGTI-2166 potently inhibit PI3K/AKT2 activation and survivin expression in both cisplatin-sensitive and -resistant human ovarian cancer cell lines. Furthermore, our data suggest a mechanism by which GGTIs repress survivin expression by showing that GGTI-298 inhibits mRNA and promoter activity of survivin independent of p53 status and AKT2 activation (Figure 8). Finally, we provide evidence that GGTI-induced apoptosis is independent of p53 pathway. Since upregulation of Akt and survivin as well as inactivation of p53 are frequently associated with chemoresistance, GGTIs could be valuable agents to overcome antitumor drug resistance. Further investigations are required to characterize the mechanism by which GGTIs downregulate survivin and inactivate PI3K, that is, identification of GGTI-298- and GGTI-2166-targeted geranylgeranylated proteins, and posi-

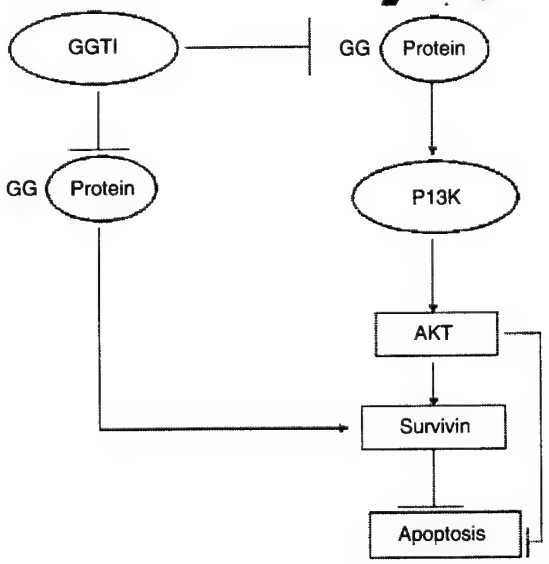

Figure 8 Schematic illustration of the mechanism of GGTI-298 induction of apoptosis in human cancer cells

tively regulate PI3K/Akt and survivin pathways independently (Figure 8).

Material and methods

Cell lines, transfection, and cell treatment

Human ovarian epithelial cancer cell lines A2780S and A2780CP and human embryonic kidney (HEK) 293 were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS. The cells were seeded in 60-mm Petri dishes at a density of 0.6×10^6 cells/dish and were transfected with 2 μg of DNA per dish using LipofecAMINE Plus. Stable clonal cell lines were established by G418 (500 μg/ml) selection.

Expression constructs

HA-AKT2 and HA-Myr-AKT2 were prepared as described previously (Jiang *et al*., 2000). HA-tagged p53 was prepared by releasing p53 from GST-p53 plasmid, kindly provided by Jiandong Chen at H Lee Moffitt Cancer Center, and cloning to HA-pcDNA3.1 vector. Survivin expression plasmid was created by PCR, subcloned to Myc-tagged pcDNA3.1 and confirmed by sequencing analysis. Based on published sequence (Li and Altieri, 1999), survivin promoter (−146/+34) was amplified by PCR using normal human placenta genomic DNA as template. The PCR products were ligated into *Bam*HI–*Sma*I sites of pGL3 vector. The promoter sequence was confirmed by DNA sequencing.

Tunel assay

Cells were seeded into 60 mm dishes and grown in 10% FBS–DMEM for 36 h. Cells were then treated with 15 μM GGTI-298 for different times ranging from 0 to 48 h. Apoptosis was determined by terminal Tunel assay using an *in situ* cell death detection kit (Boehringer Mannheim, Indianapolis, IN, USA). The cells were trypsinized, and cytospin preparations were obtained. Cells were fixed with freshly prepared paraformaldehyde (4% in PBS, pH 7.4). Slides were rinsed with PBS, incubated in permeabilization solution, followed by Tunel reaction mixture for 60 min at 37°C in a humidified chamber. After a rinse, the slides were incubated with converter-alkaline phosphatase solution for 30 min at 37°C and then detected with alkaline phosphatase substrate solution (Vector Laboratories, Burlingame, CA, USA). After an additional rinse, the slides were mounted and analysed under a light microscope. These experiments were performed in triplicate.

Immunoprecipitation, in vitro kinase assay, Western and Northern blotting analyses

Following stimulation and treatment with GGTI, cells were lysed and immunoprecipitated with anti-AKT2 or Ant-HA antibody. The immunoprecipitates were sub-

jected to *in vitro* kinase assay using histone H2B as substrate. Protein expression was determined by probing Western blots with the appropriate antibodies. For the detection of endogenous phospho-AKT2, Western blot analysis of the AKT2 immunoprecipitates was performed and detected with anti-phospho-Akt-Ser473 antibody. Detection of antigen-bounded antibody was carried out with the ECL Western Blotting Analysis System (Amersham). Northern blot was performed as previously described (Cheng *et al.*, 1992).

PI3K assay

PI3K was immunoprecipitated from the cell lysates with anti-pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl/0.1 M Tris (pH 7.4), and finally with 10 mM Tris/100 mM NaCl/1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads in reaction buffer (10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP) containing 20 μCi [-^{32}P]ATP and 10 μg L-α-phosphatidylinositol 4,5-bis phosphate (Biomol) for 20 min at 25°C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl $CHCl_3$/MeOH and phosphorylated products were separated by thin-layer chromatography as previously described (Jiang *et al.*, 2000). The conversion of PI (4,5)P_2 to PI(3,4,5)P_3 was detected by autoradiography.

Luciferase reporter assay

Cells were seeded in six-well plate and transfected with survivin-Luc reporter, pSV2-β-gal and, constitutively active of AKT2. After 36 h of transfection and treatment with or without GGTI-298, luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix), respectively. Each experiment was repeated three times.

Acknowledgements
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Activation and Overexpression of Centrosome Kinase BTAK/Aurora-A in Human Ovarian Cancer[1]

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ABSTRACT

Previous studies have demonstrated amplification of the centrosome serine/threonine kinase *BTAK/Aurora-A* in 10–25% of ovarian cancers. However, alterations of BTAK/Aurora-A at kinase and protein levels and its role in ovarian cancer progression have not been well documented. In this study, we examined the kinase activity and protein levels of BTAK/Aurora-A in 92 patients with primary ovarian tumors. *In vitro* kinase analyses revealed elevated BTAK/Aurora-A kinase activity in 44 cases (48%). Increased BTAK/Aurora-A protein levels were detected in 52 (57%) specimens. High protein levels of BTAK/Aurora-A correlated well with elevated kinase activity. Activation and overexpression of BTAK/Aurora-A were more frequently detected in early stage/low-grade ovarian tumors, although there was no statistic significance at the kinase level between early stage/low-grade and late stage/high-grade tumors. Moreover, BTAK/Aurora-A was preferentially expressed in noninvasive tumors, as revealed by immunohistochemical staining, suggesting that alterations of BTAK/Aurora-A could be an early event in human ovarian oncogenesis. To our knowledge, this is the first demonstration of recurrent activation and overexpression of BTAK/Aurora-A in human ovarian cancer, which may play a critical role in development of this malignancy.

INTRODUCTION

BTAK/Aurora-A (also named STK15, aurora-2, ARKI, and AIKI) is a serine/threonine protein kinase that belongs to the *Drosophila* aurora and *Saccharomyces cerevisiae* Ipl1 (Aurora/Ipl1p) kinase family and is essential for chromosome segregation and centrosome functions (1–3). In proliferating cells, expression of BTAK/Aurora-A is regulated in a cell cycle-dependent manner; its protein level is low in G_1-S, up-regulated during G_2-M, and reduced rapidly after mitosis (4). Immunofluorescence analysis revealed that BTAK/Aurora-A is localized to the spindle pole during mitosis, especially from prophase through anaphase (3, 4). Moreover, it has been shown that BTAK/Aurora-A interacts with Cdc20 and protein phosphatase 1 and induces cyclin B translation by phosphorylation of CPEB[4] (3) to regulate mitotic cell division (5–7). These studies suggest that BTAK/Aurora-A plays a critical role in regulation of centrosome function(s), and, thus, its alterations could result in chromosomal instability and malignant transformation. In fact, ectopic expression of BTAK/Aurora-A in Rat1 and NIH3T3 cells induces centrosome amplification, aneuploidy, and oncogenic phenotype (2, 3).

Recent studies have shown that the molecular mechanism of BTAK/Aurora-A regulation of G_2-M transition is because of phosphorylation of histone H3 (8, 9), a key molecule in conversion of the relaxed interphase chromatin to mitotic condensed chromosomes, a process likely to be essential for the subsequent nuclear division (10). Histone H3 is phosphorylated during mitosis on at least two serine residues, Ser-10 (11, 12) and Ser-28 (13, 14). Phosphorylation at Ser-10 in the histone H3 tail, which occurs early in the G_2 phase within pericentromeric heterochromatin and which by metaphase has spread throughout all chromosomal region, is considered to be a crucial event for the onset of mitosis. Phosphorylation on Ser-28 only becomes evident in early mitosis. It has been demonstrated in yeast, nematodes, and mammalian cells that BTAK/Aurora-A physically interacts with histone H3 and phosphorylates both Ser-10 and Ser-28 (8, 9). In addition, a recent report shows that BTAK/Aurora-A phosphorylates CPEB on Ser-174, which is necessary for cyclin B1 RNA polyadenylation-induced translation and entry into M phase (7). These data indicate that the Aurora kinase family plays a pivotal role during the G_2-M transition.

The *BTAK/Aurora-A* gene was mapped to human chromosome 20q13.2–13.3, a region frequently shown to be amplified in human carcinomas of breast, ovary, and colon (2). In fact, previous studies showed that the *BTAK/Aurora-A* was amplified in 15–25% of ovarian cancer cell lines and primary tumors (3, 15). In the present study, we show elevated kinase and protein levels of BTAK/Aurora-A in about half of the primary ovarian cancer specimens examined, indicating that alterations of

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[4] The abbreviations used are: CPEB, cytoplasmic polyadenylation element-binding protein; MBP, myelin basic protein; GST, glutathione *S*-transferase; HEK, human embryonic kidney; LMP, carcinomas of low malignant potential; HA, hemagglutinin.

Table 1 Alterations of BTAK/Aurora-A and tumor histopathology

Histology	*n*	BTAK protein level		BTAK kinase activity	
		Low/no	High/moderate	Low	High
LMP	10	2	8	2	8
Serous cystadenocarcinoma	58	27	31	31	27
Mucinous cystadenocar.	12	4	8	7	5
Endometrioid cystadenocar.	6	2	4	3	3
Clear cell cystadenocar.	1	1	0	1	0
Granulosa cell tumor	4	4	0	4	0
Mixed tumor	1	0	1	0	1

BTAK/Aurora-A at the kinase and protein levels are common events, which could play a pivotal role in human ovarian oncogenesis.

MATERIALS AND METHODS

Tumor Specimens, Cell Lines, Transfection, and Statistic Analysis. All of the primary human ovarian cancer specimens were obtained from patients who underwent surgery at H. Lee Moffitt Cancer Center, and each sample contained ≥80% tumor cells, as was confirmed by microscopic examination. The tissues were snap frozen and stored at −70°C. Histopathologically, the ovarian cancer specimens include 10 LMP, 58 serous, 12 mucinous, and 6 endometroid ovarian surface epithelial cystadenocarcinomas, 1 clear cell carcinoma, and 1 mix tumor. We also evaluated 4 granulosa cell tumors (Table 1). Six normal tissues adjacent to tumors and normal ovaries were used as controls. Slides from each case were reviewed for grade following the criteria of the American Joint Committee on Cancer, 1988 edition. HEK293 cells were cultured at 37°C in DMEM supplemented with 10% FCS. Transfection was carried out with calcium phosphate. The relationship between the alteration of BTAK/Aurora-A and tumor grade and stage was analyzed with χ^2 tests.

Expression Constructs, GST Fusion Protein, and Production of Anti-BTAK/Aurora-A Antibody. The pcDNA3-*BTAK/Aurora-A* was kindly provided by Dr. Subrata Sen (The University of Texas M. D. Anderson Cancer Center). We subcloned HA epitope-tagged, wild-type *BTAK/Aurora-A* (1.2 kb) at the *Not*I sites of the mammalian expression vector pHM6 (Boehringer Mannheim). The GST-BTAK/Aurora-A was created by PCR amplification of aurora box-2 of BTAK/Aurora-A (2) using primers 5′-CAGGCTCAGCGGGTCTTGTGTC-3′ and 5′-CAGTTCCTCCTCAGGATT-3′. The PCR products were inserted into pGEX-4T vector. Logarithmically growing cultures of *Escherichia coli* TOP10 transformed with the pGEX-4T recombinant was incubated with 0.1 mM isopropyl-D-thiogalactopyranoside at 37°C for 6 h. The cells were pelleted, resuspended in cold PBS, and sonicated on ice. Debris was removed by centrifugation, and the supernatant was applied to a glutathione-sepharose 4B column (Pharmacia Biotech). GST-BTAK/Aurora-A fusion protein was eluted. Anti-BTAK/Aurora-A antibodies were raised in New Zealand White rabbits. Approximately 300 μg of GST fusion protein were used to immunize each rabbit every 2 weeks; rabbits were bled 12 days after each booster injection. The antibodies were affinity purified.

Immunoprecipitation and Western Blotting Analyses. The frozen tissue was lysed by a Tissue Tearor in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% NP40, 5 mM EGTA (pH 7.5), 1 mM EDTA (pH 8.0), 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 10 mM NaF, 10 mM $NaPP_i$, 1 mM sodium vanadate, and 25 mM β-glycerolphosphate. Lysates were centrifuged at 12,000 × *g* for 15 min at 4°C. Equal amounts of protein lysate were analyzed for BTAK/Aurora-A expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4°C for 20 min. After removal of the beads by centrifugation, lysates were incubated with anti-BTAK/Aurora-A polyclonal antibody in the presence of 30 μl of protein A:protein G (2:1) agarose beads (Life Technologies, Inc.) for 2 h at 4°C. The beads were washed three times with the lysis buffer. Protein expression was determined by Western blotting analyses probed with anti-BTAK/Aurora-A or anti-HA antibody. Detection of antigen bound antibody was carried out with the ECL Western Blotting Analysis System (Amersham).

***In Vitro* Protein Kinase Assay.** The immunoprecipitation for BTAK/Aurora-A kinase assay was performed as described above. The beads were washed three times with lysis buffer and two times with kinase buffer [100 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8), 20 mM $MgCl_2$,10 mM $MnCl_2$, and 1 mM DTT] in the presence of the protease inhibitors. The reaction was carried out with 10 μCi of [^{32}P]ATP and 3 μM unlabeled ATP in 30 μl of kinase buffer. MBP (4 μg) was used as exogenous substrate. After incubation at 37°C for 30 min, the reaction was stopped by adding protein-loading buffer and separated by SDS-PAGE. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantified with a PhosphorImager (Molecular Dynamics).

Southern and Northern Blotting Analyses. Genomic DNA and total RNA were isolated from the human ovarian tumor specimens by standard methods (2). Southern blots were prepared by digestion of 10 μg of DNA with EcoRI and detected with a random primer [^{32}P]dCTP-labeled *BTAK/Aurora-A* cDNA probe. Autoradiographs were quantified relative to β-actin using Image-Quant software. For Northern blotting analyses, 20 μg of total RNA were electrophoresed on agarose gel, transferred, and detected with *BTAK/Aurora-A* cDNA probe.

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven after

dewaxing and rehydration. The Vectastain ABC Kit for rabbit IgG (Vector Laboratories) was used to immunostain the tissue sections with anti-BTAK/Aurora-A antibody. Endogenous peroxidase and biotin were blocked, and sections were incubated 1 h at room temperature with a 1:250 dilution of antibody to BTAK/Aurora-A. The remainder of the staining procedure was performed according to the manufacturer's instructions using diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin for counterstaining. Primary antibody was replaced with an equal concentration of nonimmune rabbit IgG on control sections.

RESULTS

Characterization of BTAK/Aurora-A Antibody. Previous studies have demonstrated amplification of the *BTAK/Aurora-A* in 10–25% of human ovarian cancer (3, 15). However, alterations of BTAK/Aurora-A at kinase and protein levels have not been documented. To examine BTAK/Aurora-A kinase activity and protein expression in human ovarian carcinoma, we generated rabbit polyclonal anti-BTAK/Aurora-A antibody. The specificity of the antibody was examined with HEK293 cells transfected with the HA-*BTAK/Aurora-A* expression plasmid. Western blotting analyses revealed that anti-BTAK/Aurora-A antibody reacted strongly with BTAK/Aurora-A protein (Fig. 1*A*). After preincubation of anti-BTAK/Aurora-A antibody with GST-BTAK/Aurora-A antigen, no BTAK protein was detected (Fig. 1*B*). In addition, we have further examined the usefulness of the anti-BTAK/Aurora-A antibody for immunoprecipitation. Immunoprecipitates were prepared with anti-BTAK/Aurora-A antibody in HEK293 cells transfected with the HA-*BTAK/Aurora-A* expression construct, separated in SDS-PAGE, and detected with anti-HA antibody. As shown in Fig. 1*C*, anti-BTAK/Aurora-A antibody is capable of precipitating BTAK/Aurora-A protein from the cell lysate. These results indicate that our anti-BTAK/Aurora-A antibody specifically reacts with BTAK/Aurora-A and works for both Western blot and immunoprecipitation.

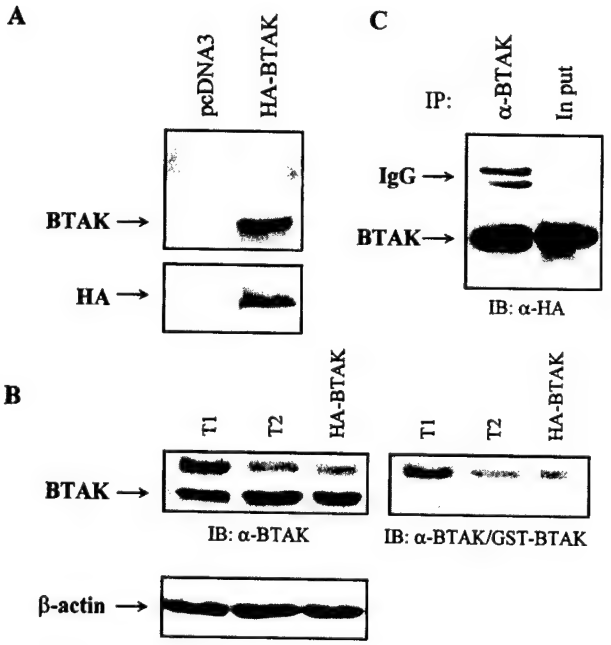

Fig. 1 Characterization of anti-BTAK/Aurora-A antibody. In *A*, BTAK/Aurora-A antibody reacts with high levels of BTAK/Aurora-A protein. HEK293 cells were transfected with pcDNA3 or pcDNA3-HA-tagged *BTAK/Aurora-A* expression plasmid. Cell lysates were subjected to Western blotting analyses with anti-BTAK/Aurora-A (*top*) or anti-HA (*bottom*) antibody. Endogenous BTAK/Aurora-A in HEK293 cells is undetectable (*left lane* of *top panel*). In *B*, anti-BTAK/Aurora-A antibody specifically reacts with BTAK/Aurora-A. Two tumor specimens overexpressing BTAK/Aurora-A- and HA-*BTAK/Aurora-A*-transfected HEK293 cell lysates were detected with anti-BTAK/Aurora-A antibody (*top left panel*). The same blot was probed with anti-BTAK/Aurora-A serum preincubated with GST-BTAK/Aurora-A antigen (*top right panel*) or β-actin (*bottom*) antibody. In *C*, the BTAK/Aurora-A antibody is able to immunoprecipitate BTAK/Aaurora-A protein. HA-*BTAK/Aurora-A*-transfected HEK293 cells were lysed and incubated with anti-BTAK/Aurora-A antibody in the presence of protein A:G. The immunoprecipitates were subjected to immunoblotting analyses with anti-HA antibody.

Frequent Activation of BTAK/Aurora-A Kinase in Primary Ovarian Tumors. As BTAK/Aurora-A is a serine/threonine kinase and plays a significant role in cell proliferation by phosphorylation of downstream targets, such as histone H3 and CPEB (7–9), we have examined BTAK/Aurora-A kinase activity in human primary ovarian carcinoma. *In vitro* BTAK/Aurora-A kinase assays were performed in 92 specimens using MBP as substrate. Fig. 2*A* shows that the *in vitro* kinase conditions that we used could detect high levels of BTAK/Aurora-A kinase activity in *BTAK/Aurora-A*- but not pcDNA3-transfected HEK293 cells. Activation of BTAK/Aurora-A, defined as an average reading of the kinase activity 4-fold higher than that in normal ovarian tissues, was detected in 44 (48%) ovarian cancer specimens. The results were confirmed by triplicate experiments. Fig. 2*B* represents typical examples of BTAK/Aurora-A activation in ovarian tumors.

In addition, we have examined the relationship between kinase activity and protein level of BTAK/Aurora-A. Western blot and immunohistochemical staining analyses revealed that all of the 44 cases with elevated levels of BTAK/Aurora-A kinase overexpressed BTAK/Aurora-A, indicating that activation of BTAK/Aurora-A is largely attributable to increased expression levels of BTAK/Aurora-A protein. To determine whether elevated protein levels of BTAK/Aurora-A result from its alterations at DNA and/or mRNA levels, we performed Northern and Southern blotting analyses in 43 tumor specimens and 6 normal ovarian tissues. Overexpression of *BTAK/Aurora-A* mRNA was detected in 18 (42%) tumors, in which BTAK/Aurora-A protein was elevated, whereas amplification of the *BTAK/Aurora-A* (>3-fold higher than that in normal ovary) was only observed in 6 (14%) specimens, all of which also exhibited overexpression of the *BTAK/Aurora-A* mRNA and protein. Representative examples are shown in Fig. 3, *B* and *C*, suggesting that BTAK/Aurora-A could be regulated at transcription, translation, and/or post-translational levels.

Elevated Expression of BTAK/Aurora-A Protein in Human Ovarian Carcinomas. We next examined the expression of BTAK/Aurora-A protein in the same series of human ovarian tumors. Western blotting analyses revealed high levels of BTAK/Aurora-A protein in 52 of 92 (57%) tumor specimens (Fig. 3*A* and Table 1). To confirm this result, we have carried out immunohistochemical staining of paraffin sections with anti-BTAK/Aurora-A

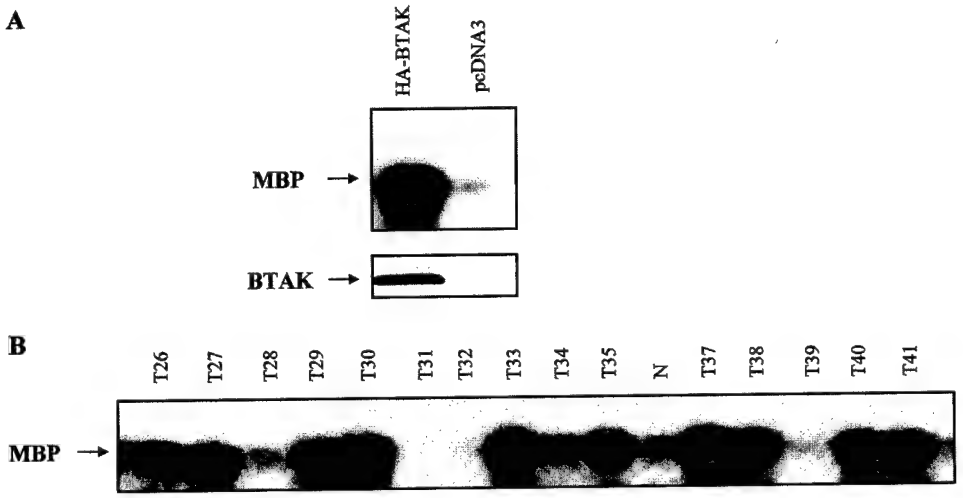

Fig. 2 Activation of BTAK/Aurora-A in human ovarian cancer. *A, in vitro* kinase assay analysis of BTAK/Aurora-A immunoprecipitates prepared from *BTAK/Aurora-A*- (*left*) and pcDNA3 (*right*) -transfected HEK293 cells with anti-BTAK/Aurora-A antibody. MBP was used as substrate (*top*). *Bottom panel,* Western blot probed with anti-BTAK/Aurora-A antibody. *B, in vitro* kinase assays of BTAK/Aurora-A immunoprecipitates from 15 representative frozen ovarian tumor specimens and a normal ovarian tissue (*N*). Tissue lysates were incubated with anti-BTAK/Aurora-A antibody in the presence of protein A-protein G agarose beads for 2 h. After extensive washes, immunoprecipitates were subjected to *in vitro* kinase assay using MBP as the exogenous substrate. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a PhosphorImager.

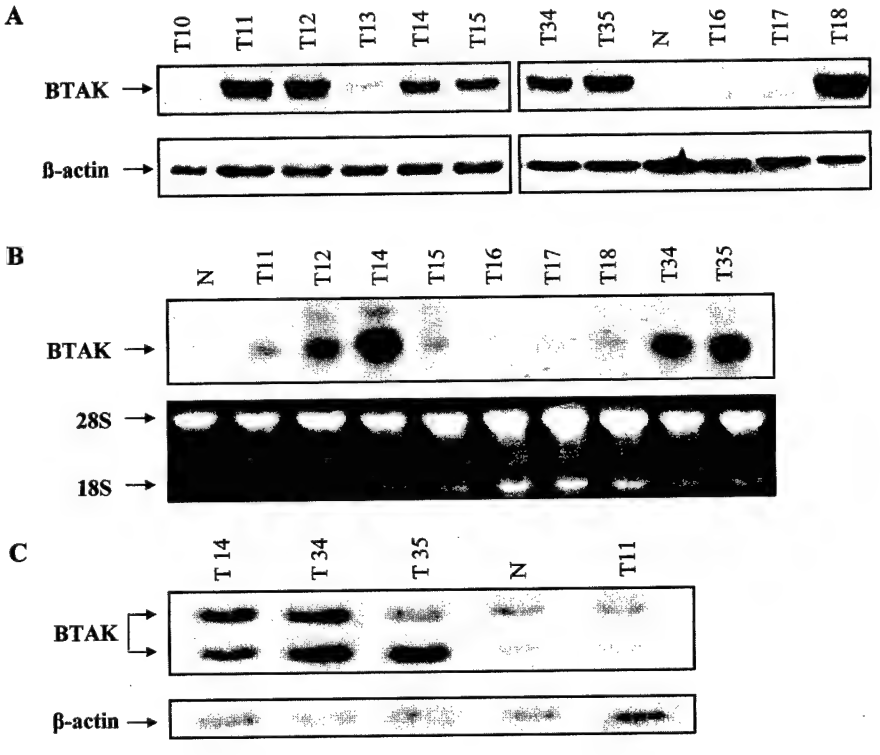

Fig. 3 Overexpression of BTAK/Aurora-A in ovarian carcinoma. *A,* Western blotting analyses of human primary ovarian tumor specimens (*T*) and normal ovarian tissue (*N*). Sixty μg of protein from each specimen were separated in SDS-PAGE, transferred, and detected with anti-BTAK/Aurora-A antibody (*top*). A weak low molecular weight band detected in N, T16, and T17 could be an alternatively spliced form because preincubation of BTAK/Aurora-A antibody with GST-BTAK/Aurora-A antigen is able to compete this band (data not shown). Equal loadings were indicated by hybridization of the same blot with anti-β-actin antibody (*bottom*). *B,* Northern blotting analyses of expression of *BTAK/Aurora-A* mRNA with [^{32}P]-dCTP-labeled *BTAK/Aurora-A* cDNA as probe (*top*). The quality and loading amount of RNA were indicated by 28S and 18S ribosome RNA (*bottom*). In *C,* Southern blots containing 10 μg of *Pst*I-digested DNA per lane from primary human ovarian tumors and a normal ovary control were hybridized with a *BTAK/Aurora-A* cDNA probe. The location of the 2.8 and 2.5 kb of *BTAK/Aurora-A* fragments is marked. Blot was stripped and reprobed with a human β-actin probe to confirm equal loading.

antibody. A moderate to strong predominantly cytoplasmic BTAK/Aurora-A expression was detected in the same 52 ovarian tumors (Fig. 4) that overexpress BTAK/Aurora-A protein revealed by Western blotting analyses. It has been shown that although BTAK/Aurora-A protein localizes to both the cytoplasm and nucleus, it is mainly in cytoplasm (16). Strong immunoreaction of BTAK/Aurora-A was observed in tumor cells but not normal ovarian epithelium. There was no preferential BTAK/Aurora-A expression among the three major histopathological types of ovarian surface epithelial carcinomas (serous, mucinous, and endometroid). Four

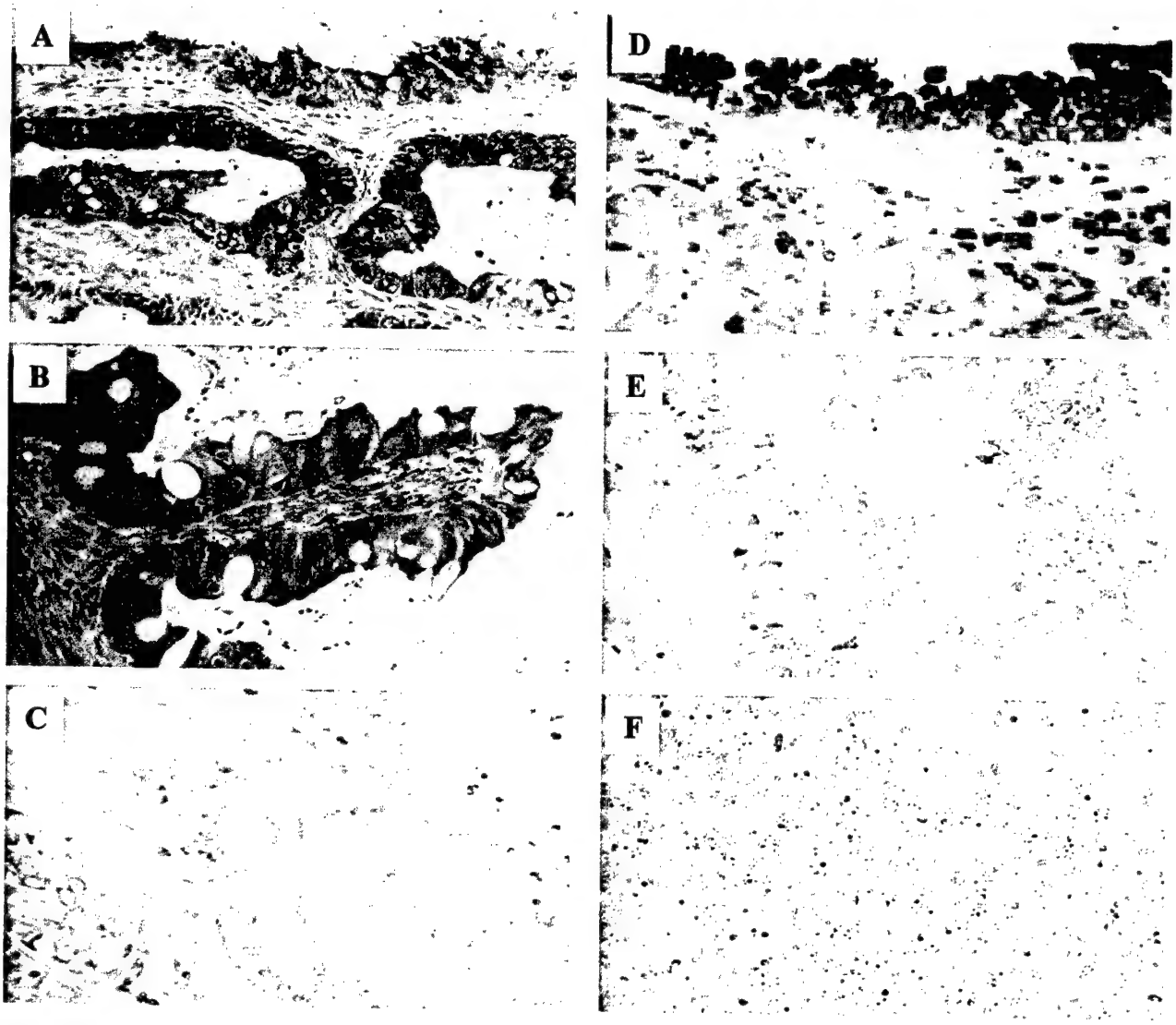

Fig. 4 High levels of BTAK/Aurora-A protein expression in low-grade and less invasive tumors. Immunohistochemical staining of the paraffin sections prepared from mucinous papillary tumors of low malignant potential (*A* and *B*), invasive mucinous adenocarcinoma (*C*), and serous adenocarcinoma (*D–F*) with anti-BTAK/Aurora-A antibody. The intensity of the immunostaining in invasive tumors is much weaker or absent in comparison with noninvasive tumors.

ovarian granulosa cell tumors exhibited no detectable BTAK/Aurora-A, implying that the expression of BTAK/Aurora-A could be restricted to ovarian epithelial neoplasm. Notably, activation/overexpression of BTAK/Aurora-A is more frequently detected in LMP (8 of 10) than serous and mucinous cystadenocarcinoma (39 of 70). Furthermore, we have observed that BTAK/Aurora-A is preferentially expressed in low-grade [25 of 39 (65%) grade I/II *versus* 13 of 33 (40%) grade III] and early stage tumors [24 of 35 (67%) stage I/II *versus* 9 of 20 (45%) stage III/IV] (Tables 2 and 3). However, there is no preference in six cases with amplification of BTAK/Aurora-A (one case is stage I, two cases are stage II, and the rest are stage III). In addition, invasive tumors exhibit much less BTAK/Aurora-A immunoreactivity compared with the noninvasive tumors (Fig. 4). Interestingly, even in the same tumor, BTAK/Aurora-A immunoreactivity was seen to pale at the invasive front of the tumor, whereas the noninvasive portion of the tumor stained strongly. Fig. 4*D* shows an invasive serous adenocarcinoma exhibiting BTAK/Aurora-A expression. However, the intensity of the staining was much less in the infiltrating component compared with the LMP component lining the surface. These data suggest that alterations of BTAK/Aurora-A could be an early event in the development of human ovarian cancer, al though there was no statistic significance at the kinase level between early stage/low-grade and late stage/high-grade tumors (Tables 2 and 3).

DISCUSSION

Ovarian cancer is thought to arise from alterations in genes involved in regulating cell proliferation, apoptosis, and genomic integrity. Alterations in several proto-oncogenes and tumor sup-

Table 2 Protein and kinase levels of BTAK/Aurora-A and tumor grade

		BTAK protein level			BTAK kinase activity		
Grade	*n*	Low/no	High/moderate	*P*	High	Low	*P*
I-II	39	14	25	<0.05	20	19	>0.05
III	33	20	13		21	12	

Table 3 Protein and kinase levels of BTAK and clinical stage

		BTAK protein level			BTAK kinase activity		
Stage	*n*	Low/no	High/moderate	*P*	High	Low	*P*
I-II	35	11	24	<0.05	15	20	>0.05
III-IV	49	27	22		30	19	

pressor genes have been described. The *ERBB2, PIK3CA,* and *AKT2* oncogenes are frequently amplified and subsequently overexpressed in ovarian cancers (17–19). Overexpression of ERBB2 or AKT2 correlates with poor prognosis of the patients (17, 18). Amplification of the *MYC* oncogene has been detected in ~20% of ovarian cancers, more frequently in serous than in mucinous cancers (20). Other oncogenes altered in ovarian cancer include *KRAS, INT2, FMS,* and *MDM2,* but these alterations appear to be relatively uncommon (17). Cytogenetic and comparative genome hybridization studies have revealed frequent gains in chromosome 20q11–13 copy number in ovarian cancer (21). Several putative candidate oncogenes from this region have recently been identified, including *AIB3* and *AIB4* mapping to 20q11, *AIB1* gene at 20q12, *MYBL2* and phosphotyrosine-phosphatase 1 genes at 20q13, and *ZNF217* and *BTAK/Aurora-A* genes at 20q13.2 amplicon (3, 15, 22). Amplification of the *BTAK/Aurora-A* has been reported in 15–25% ovarian cancer cell lines and primary tumors (3, 15). In the present study, we have studied kinase activity and protein expression of BTAK/Aurora-A in primary ovarian carcinomas. Elevated kinase activity and protein levels of BTAK/Aurora-A were detected in 48 and 57% of the tumors examined, respectively. These data indicate that alterations of BTAK/Aurora-A at kinase and protein levels are frequent changes in human ovarian cancer. Thus, BTAK/Aurora-A could play a pivotal role in the pathogenesis in the majority of cases of this malignancy.

We have also examined DNA amplification and mRNA overexpression of the *BTAK/Aurora-A* in 43 tumors. Frequency of alterations of the *BTAK/Aurora-A* at DNA level is much lower than at protein and kinase levels. Moreover, overexpression of *BTAK/Aurora-A* mRNA is much more common than amplification of *BTAK/Aurora-A*. A possible reason for these findings is insensitive detection of amplification by Southern blot analyses. Interphase fluorescent *in situ* hybridization has been reported to be more sensitive for detection of gene amplification. A recent report using fluorescent *in situ* hybridization demonstrated amplification of *BTAK/Aurora-A* in 6 of 24 (25%) sporadic ovarian carcinomas (15). A second possibility to explain the findings relates to the transcriptional regulation of BTAK/Aurora-A. In addition, our data show that overexpression of BTAK/Aurora-A protein is ~20% higher than overexpression of its mRNA in the human primary ovarian tumors examined, which could be because of RNA quality. However, based on 28S and 18S bands in our Northern blots, RNA degradation did not occur in any of the 43 tumors we examined (Fig. 3), suggesting that translational and/or post-translational regulation could be involved in up-regulation of BTAK/Aurora-A protein in human ovarian carcinoma.

We have also observed that BTAK/Aurora-A kinase activity correlates well with its protein expression in ovarian tumor specimens examined, except in 8 cases exhibiting increased protein but not kinase levels of BTAK/Aurora-A. We and others (23, 24) have shown previously that Akt protein kinase and Stat3 DNA-binding activities in primary tumors are closely associated with the interval time to freezing the specimen after surgical resection. The 8 ovarian tumors, which have elevated levels of BTAK/Aurora-A protein but not BTAK/Aurora-A kinase, also overexpress AKT2 protein but have low levels of AKT2 kinase activity (18), indicating that these results could be attributable to improper processing and/or storage of the specimens. Nevertheless, the majority of tumors with overexpressed BTAK/Aurora-A protein displayed increased levels of BTAK/Aurora-A kinase activity, suggesting that the activation of BTAK/Aurora-A is largely caused by overexpression of its protein in the primary ovarian tumors examined in this study.

The relationship between overexpression of BTAK/Aurora-A and tumor grade/stage is controversial. A previous study in ductal breast cancer showed that overexpression of BTAK/Aurora-A protein was independent of tumor histopathological type and lacked correlation with tumor size and lymph node metastases (16). Other studies showed that alterations of BTAK/Aurora-A associate with poor prognosis in gastric cancers and high grade/late stage in breast and bladder cancer (25–27). In the present study, however, we observed that BTAK/Aurora-A protein kinase is preferentially activated/overexpressed in low-grade and early stage ovarian cancer, as well as LMP (Tables 1–3), although there was no statistic significance at the kinase level between low-grade/early stage and high-grade/late stage tumors. Moreover, immunohistochemical staining showed that BTAK/Aurora-A is preferentially expressed in less invasive tumors and declines once a tumor becomes invasive (Fig. 4, *D–F*). A recent report using a well-established rat mammary cancer model demonstrated that amplification of the *BTAK/Aurora-A* is an early genetic change during the development of

rat mammary carcinoma (28). We have also documented that ectopic expression of BTAK/Aurora-A significantly induces telomerase activity, which is required for cell immortalization and transformation.[5] Therefore, activation and overexpression of BTAK/Aurora-A protein kinase may represent early changes and play an important role in development of a subset of human ovarian cancers.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Jin Q. Cheng	Associate Professor

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
Capital Medical University, Beijing, P.R.C.	M.D.	1986	Medicine
Paris University XIII, Paris, France	Ph.D	1994	Molecular Cell Biology
Fox Chase Cancer Center, Philadelphia	Postdoc.	1996	Molecular Tumor Genetics

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Professional Experience:

1994-1996 **Postdoctoral Associate**, Fox Chase Cancer Center, Philadelphia, PA
1996-1997 **Staff Scientist**, Fox Chase Cancer Center, Philadelphia, PA
1997-2001 **Assistant Professor**, H. Lee Moffitt Cancer Center and Research Institute, Department of Pathology, University of South Florida College of Medicine, Tampa, FL
2001-present **Associate Professor,** H. Lee Moffitt Cancer Center and Research Institute, Department of Pathology, University of South Florida College of Medicine, Tampa, FL

Honors and Awards:

1997-2003 First Award from National Institute of Health
1994-1997 Special Fellow Award from The Leukemia Society of America
1994 Young Investigator Award of the International Association for the Study of Lung Cancer (IASLC),
1988-1990 Fellowship for pre-doctoral training, French Cancer Research Association (L'ARC France)

Publications (Partial Listing):

Cheng, J.Q., Godwin, A.K., Bellacosa, A., Taguchi,T., Franke, T.F., Hamilton, T.C., Tsichlis, P.N., Testa, J.R. *AKT2*, a putative oncogene encoding a member of a novel subfamily of serine-threonine protein kinases, is amplified in human ovarian carcinomas. *Proc. Natl. Acad. Sci. USA* 89:9267-9271, 1992.

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Cheng, J.Q., Ruggeri, B., Klein, W.M., Sonoda, G., Altomare, D.A., Watson, D.K. and Testa J.R. Amplification of *AKT2* in human pancreatic cancer cells and inhibition of *AKT2* expression and tumorigenicity by antisense RNA. *Proc. Natl. Acad. Sci. USA* 93:3636-3641, 1996.

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Dan HC, Coppola D, Jinag K, Liu A, Hamilton AD, Nicosia SV, Sebti SM, **Cheng J.Q.** Phosphatidylinositol-3-OH Kinase/Akt and Survivin Pathways as Critical Targets for Geranylgeranyltransferase I Inhibitors Induced Apoptosis. *Oncogene, In press*

Leung BM, Fraser M, Yan X, Dan HC, **Cheng J.Q,** Tsang BK. Akt and p53 Are Determinants of Xiap-Mediated Chemoresistance in Human Ovarian Cancer. *Cancer Res., In press*

Sun M, Yang L, Feldman RI, Sun X, Jove R, Nicosia SV, **Cheng J.Q.** Activation of Phosphatidylinositol-3-OH Kinase/Akt Pathway by Androgen through interaction of p85α, AR and Src. *J. Biol. Chem. In press*

Yang H., Ou C, Feldman FI, Kruk PA, Nicosia SV, **Cheng J.Q.** Aurora-A Upregulates Human Telomerase Reverse Transcriptase through Activation of c-Myc Transcription. *Cancer Res. Accepted*

Research Projects Ongoing or Completed During the Last 3 Years:

"AKT1 oncogene in carcinogenesis"
Principal Investigator: Jin Q. Cheng
Agency: National Cancer Institute
Type: NIH R01, CA089242
Period: June 1, 2001-May 31, 2006
The goals of this proposal are to examine the importance of AKT1 in prostate carcinogenesis and effects of AKT1-interaction protein, APαB, on AKT1 signaling and AKT1 phosphorylation of p21 on cell cycle control as well as AKT1 as a target for cancer intervention.

"AKT2 oncogene and human oncogenesis"
Principal Investigator: Jin Q. Cheng
Agency: National Cancer Institute
Type: NIH R01, CA77935
Period: June 1, 2003-May 31, 2008

The goals of this proposal are to elucidate the normal cellular function of AKT2 and thus to better understand AKT2 in human oncogenesis.

"AKT survival pathways and FTI-induced apoptosis"
Principal Investigator: Said M Sebti; Co-PI: Jin Cheng
Agency: National Cancer Institute
Type: NIH R01, CA85709
Period: 2001.1-2006.1
The goals of this proposal are to elucidate the normal AKT pathway in FTI induced apoptosis.

"AKT2 oncogene and phosphoinositide 3-kinase in breast cancer"
Principal Investigator: Jin Q. Cheng
Agency: Department of Defense
Type: Idea Award, DAMD17-01-1-0394
Period: September 1, 2001-August 31, 2004
The goals of this proposal are to examine the importance of activation of AKT2 and PI3K in drug and tamoxifen resistance.

"AKT2 oncogene and its associated protein, APBP, in human ovarian cancer"
Principal Investigator: Santo V. Nicosia (PO1); Jin Q. Cheng (Project 1)
Agency: Department of Defense
Type: PO1 Award, DAMD17-02-1-0671
Period: September 1, 2002-August 31, 2006
The goals of this proposal are to examine AKT2 and AKT2-interaction protein APBP in ovarian carcinogenesis